THE UNIVERSITY *of* EDINBURGH

Isolation, Characterisation and Differentiation of Canine Adult Stem Cells

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‘Knowledge is of no value unless you put it into practice’

Anton Chekhov (1860-1904)

Abstract

Cardiac and orthopaedic diseases are significant causes of morbidity and mortality in dogs and are therefore critical areas for veterinary research. More information regarding the pathophysiology of these diseases, and the development of novel therapeutics are sorely required and adult stem cells (ASCs) are a promising source of cells for both investigation of these diseases *in vitro* and also potentially therapeutics in the longer term.

ASCs are a readily available source of multipotent cells which bypass the ethical issues surrounding embryonic stem (ES) cells. ASCs have been described in several tissues of the body, and typically differentiate along specific cellular routes related to original source location. This thesis investigates whether ASCs can be isolated and cultured from the dog from two specific locations; cardiac, producing cardiac stem cells (CSCs); and the bone marrow, producing mesenchymal stem cells (MSCs). These cell sources will be extensively characterised at their baseline for morphology, culture behaviour and gene marker expression. Following characterisation each cell source will be subjected to differentiation techniques to examine canine ASC multipotent differentiation potential.

CSCs were isolated from cultured atrial cardiac explant tissue taken from dogs *post-mortem*, with owners' consent. These cells were able to survive successive passages in serum free media and formed large spherical cell clusters, termed 'cardiospheres'. CSCs were capable of clonal expansion under controlled culture conditions, demonstrating their ability for self-renewal. Characterisation of these cells demonstrated the expression of CSC markers; c-Kit, GATA 4 and Flk-1 and no expression of cardiac lineage markers including cardiac troponin T and I, Nkx2.5, the cardiac ryanodine receptor and the β_1-adrenergic receptor.

Primary canine MSCs were isolated from bone marrow aspirates using ficoll separation and cultured on tissue culture plastic. Canine MSCs closely resembled MSCs described from other species, such as the human and mouse, and were found to express CD44 and STRO-1 and were negative for CD34 and CD45.

CSCs and MSCs were exposed to published cardiac directed differentiation protocols and differentiation then analysed using cellular morphology and gene expression. Canine

CSCs appeared to differentiate partially along cardiac lineages with upregulation of cardiac troponin T and Nkx2.5, and down regulation of c-Kit and endothelial lineage markers. Canine MSCs demonstrated some morphological changes during cardiac differentiation, and demonstrated up-regulation of Nkx2.5 and Flk-1 but no significant alteration in other markers examined. This suggested that cardiac directed differentiation was not as successful with canine MSCs compared to CSCs and conflicting with published data using rodent MSC models.

Murine MSCs were used as a positive control cell line for cardiac directed differentiation, based upon published literature. Critically there were key marker expression differences between baseline murine and canine MSCs, including the expression of cardiac markers such as cardiac troponin T and I, and the Ryanodine receptor. Furthermore, expression analysis of cardiac genes changed with time in culture and passage number and no significant alteration was seen when cells were subjected to the cardiac differentiation protocol; thereby bringing into question the data regarding successful cardiac differentiation using murine MSCs.

Canine MSCs were further differentiated toward a chondrocyte lineage to investigate the use of MSCs for orthopaedic research. Canine MSCs were successfully differentiated toward articular type cartilage, with demonstration of extracellular matrix secretions, an upregulation of collagen type II with downregulation of collagen type I and the development of SOX9 expression in differentiated cells.

This thesis builds the groundwork for future ASC research in the dog. Successful isolation and culture of two ASC sources from the dog is demonstrated. Cardiac and cartilage directed differentiation was successful using primary sourced cells, but differentiation was found to be limited to highly specific routes for each stem cell source. The results presented here highlight the importance of analysing baseline stem cells extensively prior to differentiation and in particular, before making comparisons between cell populations isolated from different locations and species.

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I want to dedicate this thesis to those I love, whose support I couldn't be without, and those I have lost, who have made me who I am today.

Declaration

I declare that this thesis has been composed by myself and has not been submitted for any other degree. The work described herein is my own except where otherwise stated and all work of other authors is duly acknowledged.

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List of Abbreviations

Measurements

°C	degrees Celsius
L	Litre
mL	millilitre
µL	microlitre
mm	millimetre
µm	micrometre
nm	nanometre
mg	milligram
µg	microgram
ng	nanogram
mmol	millimole
µmol	micromole
nmol	nanomole
pmol	picomole
g	acceleration due to gravity

Abbreviations

3D	3-Dimensional

A

α-MHC	α-Myosin Heavy Chain
α-SA	α-Sarcomeric Actin
ACDC	Adiponectin
ANP	Atrial Natriuretic Peptide

APOD	Apolipoprotein D
ARVC	Arrythmogenic Right Ventricular Cardiomyopathy
ASC	Adult Stem Cell
Ascl1	Achaete-scute complex homolog 1
ATP	Adenosine Triphosphate
AV Node	Atrioventricular Node
5'AZA	5' Azacytidine

B

β-MHC	β-Myosin Heavy Chain
Beta 1 Ad	β1-Adrenergic Receptor
B1 Ad	β1-Adrenergic Receptor
bFGF	basic Fibroblast Growth Factor
BMP 2	Bone Morphogenic Protein 2
BMP 4	Bone Morphogenic Protein 4
bp or BP	Base Pair
bpm	beats per minute
Brn2	POU domain, class 3, transcription factor 2/Oct 7
Bry	Brachyury
BSA	Bovine Serum Albumin
BSAVA	British Small Animal Veterinary Association
Buffer RLT	Buffer from RNeasy Minikit
Buffer RDD	Buffer from RNeasy Minikit
Buffer RW1	Buffer from RNeasy Minikit
Buffer RPE	Buffer from RNeasy Minikit
BVM&S	Bachelor of Veterinary Medicine and Surgery

C

cAMP	cyclic AMP
CCL2-6, 20, 26	Chemokine (C-C motif) ligand
CD11b	Cluster of Differentiation 11b (Integrin alpha M (ITGAM))
CD14	Cluster of Differentiation 14
CD19	Cluster of Differentiation 19 (B-lymphocyte antigen)
CD29	Cluster of Differentiation 29 (integrin, beta 1 (fibronectin receptor))
CD31	Cluster of Differentiation 31 (platelet/endothelial cell adhesion molecule (PECAM 1))
CD34	Cluster of Differentiation 34
CD44	Cluster of Differentiation 44
CD45	Cluster of Differentiation 45 (protein tyrosine phosphatase, receptor type, C (PTRPC))
CD73	Cluster of Differentiation 73 (5'-nucleotidase (5'-NT))
CD79α	Cluster of Differentiation 79α
CD90	Cluster of Differentiation 90 (Thy-1)
CD105	Cluster of Differentiation 105 (Endoglin, part of the TGFβ receptor complex)
CD106	Cluster of Differentiation 106 (vascular cell adhesion molecule 1 (VECAM 1))
CD117	Cluster of Differentiation 117 (c-Kit)
cDNA	complementary DNA
Cdx2	Caudal type homeobox transcription factor 2
CEM	Cardiac Explant Media
cES Cell	Canine Embryonic Stem Cell
C-Kit	Tyrosine-protein Kinase Kit
C-Myc	Proto-oncogene c-Myc
CNX 43	Connexin 43
CNXN 43	Connexin 43
CO_2	Carbon Dioxide

COL	Collagen
Col1A2b	Collagen type I alpha 2 chain
Col2A1	Collagen type II alpha 1
Col10A1	Collagen type X alpha 1
CPM	Carboxypeptidase M
CSC	Cardiac Stem Cell
CTI	Cardiac Troponin I
CTT	Cardiac Troponin T
CXC	C-X-C Chemokin Motif
CXCR4	C-X-C Chemokine Receptor type 4
CX3CL1	C-X3-C Chemokine Ligand type 1
CXCL1-12	C-X-C Chemokine Ligand 1-12

D

D17	Canine Osteosarcoma Cell Line
D17Ad	Adherent Canine Osteosarcoma Cell Line
D17Sp	Sphere Forming Canine Osteosarcoma Cell Line
DAB	3,3' Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DCM	Dilated Cardiomyopathy
DEPC	Diethylpyrocarbonate
DEM	Differentiation Enabling Media
DKK1	Dickkopf-related protein 1
DMEM	Dulbecco’s Modified Eagle Medium
DMEM F12	Dulbecco’s Modified Eagle Medium; Nutrient Mixture F12
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNAse	Deoxyribonuclease
dNTP	deoxyribonucleotide

DPT	Dermatopontin
DPX	mixture of Distyrene, a plasticizer, and Xylene
DTT	Dithiothreitol

E

ECC	Embryonal Carcinoma Cell
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
END-2	Endoderm-like cell line 2
ES Cell	Embryonic Stem Cell
ETS-1	Erythroblastosis virus E26 oncogene homolog 1

F

FABP5	Fatty Acid Binding Protein 5
FACS	Fluorescent Activated Cell Sorting
FAM Probe	6-carboxyfluorescein Probe
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
FHDO-7	Canine ES cell line
FITC	Fluorescein Isothiocyanate
Flk-1	Fetal liver kinase 1 (kinase insert domain receptor (KDR), VEGFR2)
Flt-1	Fms-related tyrosine kinase 1 (VEGFR1)

G

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GATA 1-6	Family of Transcription Factors
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Colony Stimulating Factor 2 receptor, alpha, low-affinity (granulocyte-macrophage)
GSK-3α	Glycogen Synthase Kinase 3 alpha
GSK3-β	Glycogen Synthase Kinase 3 beta

H

hES Cell	Human Embryonic Stem Cell
HCl	Hydrochloric Acid
HCM	Hypertrophic Cardiomopathy
HERG	Potasssium Voltage-gated Channel, Subfamily H (eag-related), Member 2
HGF	Hepatocyte Growth Factor
HIER	Heat Induced Epitope Retrieval
HLA-DR	Major Histocompatibility Complex, class II, DR
HLA-G5	Major Histocompatibility Complex, class I, G
H_2O	Water
HPSF	High Purity Salt Free
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase

I

i_{ca}	inward calcium current
IDO	Indoleamine 2,3-Dioxygenase
IFA	Immunofluorescence
IGF-1	Insulin-like Growth Factor-1
IgG	Immunoglobulin G

IgM	Immunoglobulin M
IL-6	Interleukin-6
IMDM	Media
iNOS	inducible Nitric Oxide, critical for immune defense
iPS Cell	Induced Pluripotent Stem Cell
Isl1	Islet 1
ITS+	Insulin Transferrin Selenium +

J

JAK	Janus Kinase Pathways
JNK	Jun N-terminal Kinases

K

KCl	Potassium Chloride
KCNQ1	Potassium Voltage-gated Channel, KQT-like Subfamily, Member 1
KCNE1/2	Potassium Voltage-gated Channel, Isk-related family, Member 1/2
kDa	kilo Dalton
KDR	Kinase Insert Domain Receptor
Ki-67	Cellular proliferation gene (MKI67)
KitL	c-Kit Ligand
KLF4	Kruppel-like Factor 4

L

LDL	Low Density Lipoprotein
LH	Luteneising Hormone

LIF	Leukaemia Inhibitory Factor
Lin 28	Marker of undifferentiated ES cells
LQTS	Long QT Syndrome
LV	Left Ventricle
Ly6	Leucocyte Antigen 6

M

M-199	Media
MACS	Magnetic Activated Cell Sorting
MCM	Methylcellulose Semisolid Medium
MCP-1	Zinc Finger Protein, associated with apoptosis of cardiomyocytes
M-CSF	Macrophage Colony Stimulating Factor
2-ME	2-Mercaptoethanol
mES Cell	Mouse Embryonic Stem Cell
MEF	Mouse Embryonic Fibroblast
MEF2A	Myocyte Enhancer Factor 2A
MEF2C	Myocyte Enhancer Factor 2C
MEF2D	Myocyte Enhancer Factor 2D
MEK	serine/threonine-selective protein kinases
MEM	Media
MEM-NEA	MEM Non-essential Amino Acids
Mesp1	Mesoderm Posterior 1 homolog, important for development of cardiac mesoderm
$MgCl_2$	Magnesium Chloride
MLC-2a	Myosin Light Chain-2 atrial
MLC-2v	Myosin Light Chain-2 ventricular
MMP	Matrix Metalloprotease
MRI	Magnetic Resonance Imaging
MRCVS	Member of the Royal College of Veterinary Surgeons

MSc	Master of Science
MSC	Mesenchymal Stem Cell
MyBP-C	Myosin Binding Protein-C
Myt1l	Myelin Transcription Factor 1-like, neuronal differentiation
MVMD	Myxomatous Mitral Valve Disease

N

NaF	Sodium Flouride
NCBI	National Center for Biotechnology Information
Nkx2.5	NK2 transcription factor related, locus 5, Homeobox-containing Transcription Factor
NOD/SKID Mouse	Immunodeficient Mouse
NP40	Nonyl Phenoxypolyethoxylethanol 40, detergent.

O

OA	Osteoarthritis
Oct 4	Octamer-binding protein 4, POU class 5 homeobox 1
Oct 4A, Oct 4B, Oct 4B1	Splice variants of Oct 4
OFT	Outflow Tract
OMD	Osteomodulin

P

Pax 6	Paired box gene 6
PBS	Phosphate Buffered Saline
PBST	PBS Tween
PCR	Polymerase Chain Reaction

PDGF	Platelet Derived Growth Factor
PET/CT	Positron Emission Tomography – Computed Tomography
PGE-2	Prostoglandin E2, mediator in the immune effects of MSCs
pH	power of Hydrogen
PhD	Doctor of Philosophy
PI3K/AKT	Phosphatidylinositol 3-kinases/ serine/threonine protein kinase
PKA	Protein Kinase A

R

R^2	Coefficient of determination
RCVS	Royal College of Veterinary Surgeons
R(D)SVS	Royal (Dick) School of Veterinary Studies
RNA	Ribonucleic Acid
18s rRNA	18s Ribosomal RNA
RNAse	Ribonuclease
RNCM	Rat Neonatal Cardiomyocytes
rpm	revolutions per minute
RPMI 1640	Media
RT	Reverse Transcription
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
RV	Right Ventricle
RyR	Cardiac Ryanodine Receptor

S

SAN	Sinoatrial node.
SA Node	Sinoatrial node.
Sca 1	Stem cell antigen 1
SCF	Stem Cell Factor. C-Kit receptor (kitL ligand)

SCN5A	Sodium Channel, Voltage-gated, type V, Alpha subunit
SDF-1	Stromal cell-Derived Factor-1 (CXCL12)
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Sfrp 2	Secreted frizzled-related protein 2
SMA	Smooth Muscle Actin
SOX2	SRY (sex determining region Y)-box 2, maintenance of undifferentiated state
SOX9	SRY (sex determining region Y)-box 9, chondrocyte differentition
SR	Sarcoplasmic Reticulum
SSEA 1	Stage Specific Embryonic Antigen 1
SSEA 3	Stage Specific Embryonic Antigen 3
SSEA 4	Stage Specific Embryonic Antigen 4
STAT 3	Signal Transducers and Activators of Transcription 3
STRO-1	cell surface protein expressed by bone marrow stromal cells and erythroid precursors

T

TAE	Tris Acetate EDTA buffer
Tβ-4	Thymosin β4, protection, repair and remodelling of damaged tissues
TBST	Tris buffered saline and Tween 20
Tbx5	T-box 5, transcription factors involved in the regulation of developmental processes
Tbx18	T-box 18, transcription factors involved in the regulation of developmental processes
TGFβ1	Transforming Growth Factor β 1
Tm	Melting Temperature
TNF-α	Tumour Necrosis Factor-α
TRA-1-60	Pluripotent Stem Cell Marker

TRA-1-81	Pluripotent Stem Cell Marker

U

U	Units
UV	Ultraviolet

V

VEGF	Vascular Endothelial Growth Factor
VEGFR-2	VEGF Receptor 2 (Flk-1, KDR)
VIC/MGB Probe	qRT-PCR colour dye
vWF	von Willibrands Factor

W

WHT	Whole Heart Tissue
Wnt	Signalling pathway of embryogenesis
Wnt1l	Signalling factor in the Wnt pathway
Wnt5A	Signaling factor in the Wnt pathway
Wt1	Wilms tumour 1 gene

Z

ZNF145	Zinc Finger Protein 145, transcription factor

CHAPTER 1

General Introduction

1.1 Stem Cells

Stem cells are defined by having two characteristic properties: the ability for self-renewal, giving rise to more stem cells and the ability to differentiate into cell lineages from all three germ layers of the embryo under appropriate conditions (the endoderm, mesoderm and ectoderm) (Bajada et al., 2008). This differentiation has led to the cells being termed pluripotent - with the ability to divide into all cell types, or termed multipotent - with the ability to differentiate into multiple cell types of a limited number.

Stem cells are tightly controlled by a combination of intrinsic (intracellular) programs, and extracellular, micro-environmental signals. Recent work utilising techniques such as micro-patterning and cellular microarray have greatly increased known information of these cellular control systems. For instance, pluripotency is tightly controlled by the expression of genes such as Oct 4, Nanog and SOX2. Alongside this, the local environment to the stem cell, often termed the 'niche' has a large impact on the differentiation of the cell (Underhill and Bhatia, 2007).

Stem cells hold great promise for the field of regenerative medicine and tissue engineering, however currently there are very few true clinical applications, largely due to difficulties in optimising cell culture, and targeting cultured cells to specific tissue sites *in vivo*.

Ultimately the study of stem cell differentiation and the production of viable new tissue specific cells would enable the treatment of many diseases, such as neurological disorders, diabetes mellitus, orthopaedic disease and cardiac disease.

1.1.1 Defining Stem Cells

Stem cells have been defined as cells which are capable of self-renewal, and which have the ability to differentiate into multiple cell lineages. Stem cell marker profiles examining gene expression have been used to try to define stem cells *in vitro.* This technique has also proven useful in determining what stage of differentiation the cell may have reached. Cellular morphology, such as ability to form spheres, and behaviour in culture, including clonal expansion have also been used in combination with marker expression panels to attempt to define stem cells. However, this is a developing and challenging area; defining the marker or groups of markers which can be reliably used to define a cell as a stem cell is still being investigated. This is made more complicated by the possibility of interspecies differences and differences between different stem cell populations and stages of differentiation.

There are three key types of stem cells described; embryonic, adult and induced pluripotent stem cells. These differ in both origin and differentiation potential, but all have the possibility for usage in the future of regenerative medicine.

1.2 Stem Cell Classifications

1.2.1 Embryonic Stem Cells

In the 1970s embryos of normal mice, transplanted to an extra-uterine site, were found to form teratocarcinomas (Stevens, 1970). Cell lines derived from these tumours were termed embryonal carcinoma cell (ECC) lines, and were found to have morphological, biochemical and immunological properties in common with pluripotent embryonic cells. The behaviour of these cells closely resembled the behaviour of cells taken from the inner cell mass of the embryonic blastocyst (Martin et al., 1977). ECC lines were also able to participate in the development of normal adult mice, forming chimeras when injected into blastocysts (Martin, 1981).

Evans and Kaufman took blastocysts from 129SvE mice, and cultured them in groups of 6 within small drops of tissue culture medium under paraffin oil. Over 48 hours the blastocysts attached to the tissue culture plastic petri dishes and trophoectoderm grew out and differentiated into giant trophoblast cells. The inner cell mass subsequently grew, surrounded by endoderm, and was selectively picked and cultured. Actively proliferating colonies which resembled the ECC cells were picked out and passaged.

These cells grew rapidly in culture, supplemented with leukaemia inhibitory factor (LIF) and/or on feeder cell layers of mitotically inactive mouse embryonic fibroblasts (MEFs) and were maintained for over 30 passages *in vitro* (Evans and Kaufman, 1981). These were what would classically come to be defined as embryonic stem cells (ES cells). Desbaillets *et al* took this work further, and found that single undifferentiated ES cells separated into corresponding cell lineages dependent upon culture techniques: Single cells grown on methylcellulose semisolid medium (MCM) became erythroid, lymphoid, myeloid and endothelial cell lineages; ES cells grown on non-adhesive tissue culture plates in standard culture medium became erythroid, endothelial, neuronal and glial cell lineages; and hanging drops of 400-600 cells suspended on petri dish lids for two days, and subsequently transferred into suspension became lymphoid progenitors, spontaneously contracting cardiomyocytes, skeletal myocytes, vascular smooth muscle cells and adipocytes (Figure 1.1) (Desbaillets et al., 2000).

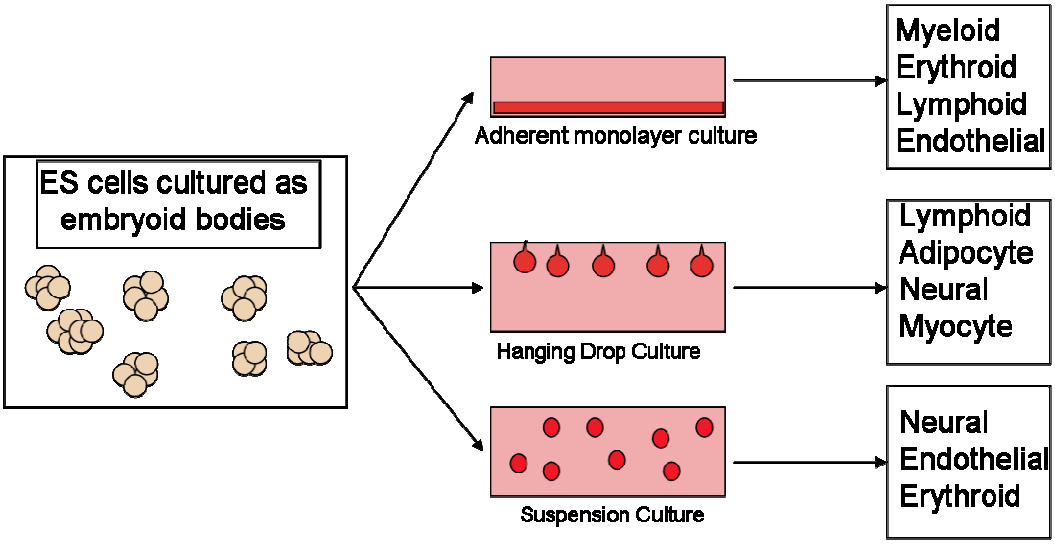


Figure 1.1. Generation of multiple cell lineages from ES cells using alternative culture techniques. Undifferentiated ES cells are disassociated into single cells and plated using three different methods, creating the initiation for differentiation into the three germ cell lineages. Adapted from (Desbaillets et al., 2000).

In 1998 Thomson *et al* described ES cells derived from human blastocysts, (human embryonic stem cells, hES cells) (Thomson et al., 1998). This work followed more than a decade of research in murine ES (mES) cells, and posed the opportunity to study developmental events which could not be studied in the intact human embryo, including comparisons of development and function of tissues between mice and humans. Initially culture of hES cells required a MEF feeder layer. Over the subsequent years more advanced systems of culturing hES cells were found, which did not need the MEF feeder layers which were a potential source of cell contamination. Instead the cells were grown

in medium with and without serum replacement, plus a combination of growth factors such as LIF, Transforming growth factor β1 (TGFβ1), basic fibroblast growth factor (bFGF) and fibronectin matrix. The hES cells in this system still maintained their ES cell properties of self-replication and pluripotency (Richards et al., 2002, Amit et al., 2004, Bajada et al., 2008).

Crucially, mES and hES cells are initially derived in different ways; mES cells are derived from culture of the inner cell mass from pre-implantation embryos. They express genes characteristic of the early blastocyst including Oct 4, SOX2 and stem cell surface markers SSEA 1 and SSEA 3, and can be grown on both a feeder layer, or without a feeder layer in the presence of LIF, removal of which promotes differentiation (Ginis et al., 2004). Interestingly, mES cells cannot differentiate into trophectoderm (Edwards, 2002). mES cells retain high telomerase levels, show karyotypic stability, and retain the ability to contribute to chimeras and teratomas after repeated passage (Burdon et al., 2002).

In contrast hES cells are derived from the inner cell mass of the post-implantation embryo (Thomson et al., 1998). These ES cells have many similarities to their mouse counterparts; they express stem cell surface markers SSEA 3 and the pluripotency gene Oct 4, as well as SSEA 4 (Mummery et al., 2003, Ginis et al., 2004). Interestingly, hES cells must be cultured in the absence of serum, which would otherwise promote differentiation (Thomson et al., 1998) and unlike mES cells these cells also have the

ability to differentiate into trophoblast-like cells and do not require the presence of LIF (Niwa et al., 2000, Schneider et al., 2010).

To date more than 400 human cell lines have been derived, giving great opportunity to gain access to hES cells (Beqqali et al., 2009). An international study characterised and compared 59 hES cell lines and found that they all had a similar expression pattern for several hES cell markers including SSEA 3 and 4, Oct 4 and Nanog, despite their different genetic background and derivation technique. However the cell lines were not identical; different expression levels in lineage markers were seen, and some gene-dependent variation was seen for imprinted genes (Adewumi et al., 2007). ES cells have been predominantly isolated from the human and mouse, however they have also been isolated from different species such as dogs (discussed later), and rabbits, rats and monkeys (Thomson et al., 1995, Wang et al., 2007, Buehr et al., 2008, Li et al., 2008).

1.2.2 Adult Stem Cells

Although ES cells were the first truly pluripotent stem cells to be identified and characterised, it was hypothesised that a mechanism existed whereby organs could repair and replenish cells as they died. The hypothesis was confirmed when it was subsequently demonstrated that many developed tissues possessed a reservoir of somatic stem cells, which still retained some of the characteristics of their ES cell predecessors, including the capacity to self-renew but had a more limited differentiation capacity, termed multipotent (Fuchs et al., 2004, Beltrami et al., 2007).

Non-embryonic stem cells are lower in the stem cell hierarchy. Their multipotent function allows them to differentiate into a limited number of cell types generally common to the tissue of origin and generally assigned to one of the three germ cell layers of the embryo (Bajada et al., 2008). Many tissues within the body have been found to contain adult stem cells (ASCs), including liver, bone marrow, brain and heart (Beltrami et al., 2007).

Cells with a multipotent differentiation potential taken from adult tissue were first studied by Becker in the 1960s; irradiated mice were injected with bone marrow cells and the subsequent colonies that formed in the spleen counted. This work suggested that the cell colonies formed were in proportion to the number of cells injected, and that each colony was derived from a single cell (Becker et al., 1963). Following this the first successful bone marrow transplant was performed between a patient suffering acute lymphoblastic leukaemia, and 6 of his immediate relatives, creating a patient with an haematopoietic chimera (Mathe et al., 1963). ASCs were subsequently formally described in the 1970s, where multiple cells from within the haematopoietic compartment were found to have derived from a common precursor – a stem cell (Fialkow, 1973). Bone marrow derived stem cells are now split into two classes, haematopoietic as previously discussed and mesenchymal, found to differentiate into multiple connective tissue types (Owen, 1988).

1.2.3 Induced Pluripotent Stem Cells

Due to the therapeutic and ethical issues surrounding ES cells (discussed in ES cells for Therapeutics), a viable alternative has been sought. In 2006 Takahashi and Yamanaka described a method whereby differentiated cells could be re-programmed to an embryonic-like state by insertion of 4 factors; Oct 4, SOX2, c-Myc and KLF4 and termed them induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006). The factors were inserted into both mES cells and adult mouse fibroblasts using a retroviral vector which subsequently over expressed the inserted factors. The following year, the same group described the successful production of iPS cells generated using human fibroblasts (Takahashi et al., 2007). iPS cells are a crucial advancement in the development of patient specific stem cells important for both individual cellular therapeutics and the study of patient specific diseases *in vitro*. iPS cells were optimised in further studies, proving that DNA methylation, gene expression and chromatin state of the iPS cells were similar to that of ES cells (Wernig et al., 2007), and were also able to produce functional germline chimeras (Okita et al., 2007). However, insertion of the oncogene c-Myc increased tumorogenicity in the chimera and progeny mice and was therefore not an ideal factor in the iPS induction system; therefore in 2008 Nakagawa *et al*, devised a method to produce functional iPS cells without c-Myc, and found that chimeric mice derived from the c-Myc negative iPS cells did not develop tumours during the period of the study (Nakagawa et al., 2008).

Another concern with the generation of iPS cells has been the use of retroviral vectors, which renders the cells unusable in a therapeutic setting. In 2009 two new techniques were described. Firstly, a *piggy*Bac technique, which enables insertion of the four original factors into both mouse and human embryonic and adult fibroblasts, generating iPS cells. The genes required for iPS transition are inserted into a transposon (a section of DNA which moves as a block within the genome) named *piggyback*; first discovered in moths and butterflies, this transposon has an innate ability of reliably and efficiently inserting itself into the genomes of mice and humans (Kaji et al., 2009, Woltjen et al., 2009). Secondly, the insertion of the 4 factors using Sendai Virus, an RNA virus which effectively produced iPS cells which could then be further selected against to remove virus carrying cells using simple antibody-mediated negative selection (Fusaki et al., 2009).

1.3 Stem Cell Niche

Stem cells are thought to reside within a 'niche'; a biological microenvironment, specific to the cell, helping to promote and maintain the stem cells' innate characteristics (Urbanek et al., 2006). This environment provides the necessary signals required to drive appropriate cell differentiation. In ES cells, the niche is well defined, being part of the embryo itself. However, since the discovery of adult stem cells within various tissues different tissue specific niches have been described. When attempting to culture adult stem cells, trying to mimic the specific cell's niche as closely as possible will promote cell self-renewal, and limit differentiation. Niches exist to protect the stem cells, and not

only include the stem cells, but a host of differentiated cell types which secrete a rich mix of extracellular matrices and other factors. There is considerable variation within different stem cell niches: for instance muscle stem cells (satellite cells) exist in relative isolation on the basal lamina of the muscle fibre bundle, and only become active when myofibres are damaged, whereas intestinal stem cells reside at the base of the intestinal crypts and are constantly active, producing replacement paneth cells, goblet cells, absorptive villus cells and extraendocrine cells (Fuchs et al., 2004).

1.4 Stem Cells and Disease

The development of stem cells has provided new opportunities for the study of the pathophysiology of disease and introduced the prospect of using stem cells for regenerative medicine, tissue engineering and therapeutics. This is a developing field and much research is still ongoing to optimise these uses.

1.4.1 Embryonic Stem Cells in Therapeutics

Using ES cells in therapeutics is potentially fraught with problems. Teratoma formation was described very early in the literature, and due to the innate ability of ES cells to differentiate into all three germ layers of the body (i.e. any tissue within the body), strict methods of controlling differentiation *in vivo* are crucial before they can be used successfully (Martin, 1981, Barile et al., 2007).

The potential use of ES cells within treatment regimes requires large scale production of a homogeneous population of differentiated cells. As previously discussed, currently > 400 hES cells have been derived worldwide (Beqqali et al., 2009). Although the significant majority of the cell lines are similar, some important differences have been demonstrated (Adewumi et al., 2007). Directed differentiation of ES cells to a specific lineage creates a mixed population of cells, each at their independent level of differentiation. Ultimately, differentiated cells derived from an ES cell source must be a homogeneous population prior to any form of transplantation to limit the significant risk of teratoma formation (Odorico et al., 2001, Laflamme et al., 2007, Beqqali et al., 2009).

Ethically, the use of ES cells has been questioned. To obtain ES cells, they must be harvested from a fertilised embryo. In all species, but most importantly in the hES cell lines, the ethics of this has been questioned, as these fertilised embryos would have the potential of forming a living creature (Bajada et al., 2008). Debate will continue to surround this sensitive subject, with the moral status of the embryo (Towns and Jones, 2004) and the potential use of saviour siblings as a source of higher matched ES cells (Spriggs and Savulescu, 2002) fuelling the arguments for and against.

Finally, implanting differentiated ES cells for specific treatment into a host would cause an immunological reaction akin to that found in transplant patients. Recipients of the ES cells would require immunosuppressive therapy to allow a more successful outcome of the implant, which in itself makes the treatment protocol more hazardous (Swijnenburg et al., 2005).

1.4.2 Adult Stem Cells in Therapeutics

ASCs have been described in multiple organs within the body (Beltrami et al., 2007). It is unknown if these cells are stem cells left over from embryonic development or whether they are a separate cell type. They have multipotent differentiation potential, usually toward cell types found at their tissue of origin. These cells are not capable of teratoma formation, and there are no ethical objections to their use as their origin is from fully developed animals, without the requirement for destruction to obtain tissue. However, one limitation in the use of ASCs is that the isolation of this cell population generally requires surgical procedures and cells are often obtained in small numbers. These cells may hold the most realistic potential for therapeutic use, as they can be used autologously and can be given with patient consent but scalability will need to be addressed. The discussion of individual ASC populations can be found in Chapters 4 to 7.

1.4.3 Induced Pluripotent Stem Cells in Therapeutics

iPS cells are capable of pluripotent differentiation (Hu et al., 2010) and circumvent some of the key issues relating to ES cells. Derived from adult somatic cells, these cells do not have the same ethical concerns surrounding usage of embryonic tissue. Furthermore, the ability to derive truly autologous stem cells should limit the patient's immunological reaction should these cells be transplanted back into the original donor. However, given

that these cells are otherwise essentially similar to ES cells, they still have the ability to form teratomas. Furthermore, c-Myc is a known oncogene and therefore use of any transformed cells carrying this gene would be impossible. However, it is hoped that patient specific iPS cells will allow the study of disease *in vitro* more readily.

1.5 The Canine Perspective

The pet dog contributes to a significant amount of cases seen in small animal veterinary practice. The use of dogs as a subject allows us to study canine specific diseases more closely, advancing the understanding of the biology of veterinary disease. Furthermore, canine research may contribute to the development of novel therapeutics which could improve treatment regimes for other veterinary species. The dog is not only a patient in its own right, but also inadvertently an ideal model for the study of human diseases. Large animal models of disease are a requirement for any translation of basic science into therapeutics and practice (Dixon and Spinale, 2009). Many diseases are known to affect both species, and in the western world the dog and human are exposed to many similar environmental factors of disease. The dog is a larger species than many commonly used rodent models, and also has greater longevity thereby allowing for age influence. Furthermore the pedigree dog is a relatively inbred model allowing for genetic influences on disease to be studied. Isolating stem cell populations from the dog will allow for *in vitro* disease modeling and may also act as an animal sparing process in drug discovery. The dog has been the prevalent species in transplantation research and is therefore known to be an essential model for human translation (Kirk, 2003). Dog

lifespan allows for longer term follow up on both cellular transplantation and gene therapy, and similarities can be seen in the molecular and physiological basis of disease, including cancer (Schneider et al., 2010).

1.5.1 Canine Embryonic Stem Cells

Canine ES (cES) cells were first developed by Hatoya *et al* in 2006 and demonstrate some similarities with hES cells (Table 1.1). Pre-implantation embryos were collected from Beagle bitches at day 11-16 of first oestrus. Inner cell masses were removed from blastocysts and cultured on MEFs in the presence of LIF whereby they formed colonies. Both enzymatic and mechanical dissociation of the inner cell mass was performed; enzymatic dissociation resulted in immediate differentiation, however mechanical dissociation created two ES-like cell lines which survived 8 passages, had stem cell morphology and expressed stem cell markers such as SSEA 1, Alkaline Phosphatase and Oct 4 and were negative for SSEA 4. The cES cells could be cultured in embryoid bodies which differentiated into several cell types including neuron-like, fibroblast-like, melanocyte-like, and myocardium-like cells (Hatoya et al., 2006). Following this cES cells were derived from a Golden Retriever bitch, grown on MEFs and were found to express stem cell markers SSEA 1, Alkaline Phosphatase, Oct 4, Nanog and SOX2, and were again negative for SSEA 4. The cES cell line derived was maintained for 10-12 passages, but continuous passages were increasingly difficult. Co-culture of these cells with murine bone marrow cells created a population of CD34 and GATA 2 positive

cells, indicating differentiation toward the haematopoietic lineage (Schneider et al., 2007, Schneider et al., 2008).

Hayes *et al* generated several blastocyst-derived cell lines, of which one, FHDO-7 could be maintained for 34 passages and expressed Oct 4, Nanog, Alkaline Phosphatase and telomerase, and a cluster of microRNAs found in human and mouse ES cells. FHDO-7 cells grew on MEFs in the presence of LIF and could form embryoid bodies which gave rise to ectoderm, mesoderm and endoderm derived cells (Hayes et al., 2008). All cES cell lines up to this point were unable to differentiate *in vivo* and therefore unable to form teratomas in immunodeficient mice. In 2009 a fourth group derived cES cells from pre-implantation embryos and found that days 12-14 post luteinising hormone (LH) surge was the optimum time for embryo collection which concurred with the previous studies consistently stating that late stage, or 'hatched' blastocysts from days 13-14 post LH surge are most efficient. These cES cells were grown on MEFs in the presence of both LIF and human bFGF. Oct 4, Nanog, SOX2 and SSEA 3 and 4 were all expressed, with SSEA 1 found at very low levels. These cells could be maintained for 30 passages and were capable of forming embryoid bodies which could subsequently be differentiated into cell lines from all three germ layers. Crucially, it was found that these cES cells could form teratomas in NOD/SKID mice (Vaags et al., 2009). Most recently canine ES cells have been found capable of synaptically-competent neuronal differentiation, giving an indication of their specific differentiation potential (Wilcox et al., 2009, Wilcox et al., 2011).

Table 1.1. Comparison of ES cell characteristics between species.

Marker	Mouse ES Cell	Human ES Cell	Dog ES Cell
SSEA 1	+	-	+/-
SSEA 3	+/-	+	+
SSEA 4	-	+	+/-
TRA-1-60	-	+	+
TRA-1-81	-	+	+
Alkaline Phosphatase	+	+	+
Oct 4	+	+	+
Feeder cell required	Yes	Yes	Yes
LIF required	Yes	No	Yes
Embryoid bodies in vitro	Yes	Yes	Yes
Teratomas in vivo	Yes	Yes	Yes/No

Adapted from (Schneider et al., 2010)

cES cells can only be derived from *ex vivo* collection of embryos; *in vitro* production is rendered technically difficult due to the canine specific pre-ovulatory increase in progesterone and ovulation of immature oocytes, therefore creating a large obstacle for the development of an *in vitro* fertilization and maturation system (Songsasen and Wildt, 2007, Schneider et al., 2008).

1.5.2 Canine Adult Stem Cells

Different ASC sources have been described in the dog. Mesenchymal stem cells (MSCs) predominate in the literature however follicular stem cells have also been described (Kadiyala et al., 1997, Kobayashi et al., 2010, Vieira et al., 2010, Zucconi et al., 2010). Furthermore, cloned Beagles have been generated from adipose derived MSCs from an original cloned dog demonstrating germline competence (Oh et al., 2011). As the field of ASCs develops more tissue specific stem cells are likely to be discovered in the dog. Cardiac and mesenchymal canine adult stem cells are discussed in further detail in Chapters 4-7.

1.5.3 Canine Induced Pluripotent Stem Cells

Canine iPS cells have been developed only relatively recently. Shimada *et al* reported the production of canine iPS cells using retroviral transduction of the four classic iPS genes (Oct 4, c-Myc, KLF4 and SOX2) into canine embryonic fibroblasts. The cultured cells were grown in the presence of LIF and TGFβ1 and growth inhibitors of MEK and GSK3-β, as described by Li *et al*, and were capable of differentiation into all three germ layers; mesoderm, ectoderm and endoderm (Li et al., 2009, Shimada et al., 2010). More recently it has been shown that canine iPS cells derived from canine adult dermal fibroblasts and adipose stromal cells require LIF and bFGF for formation and growth, but do not require the inhibition of MEK and GSK3-β. These iPS cells were also capable

of differentiation along all three germ layers (Lee et al., 2011, Luo et al., 2011). Interestingly it was shown that reprogramming of adipose derived cells was twice as efficient as that of fibroblasts, suggesting that original cell source may be relevant for successfully reprogramming, even though on microarray analysis both sources of reprogrammed cells were similar in expression profile to each other and canine ES cells (Lee et al., 2011). iPS cells are a rapidly developing field and the generation of canine iPS cells will allow for greater study of veterinary diseases *in vitro.*

This general introductory chapter is followed by Chapter 2 which describes general cellular and molecular techniques which are common to all sections of the thesis. Following this is Chapter 3, which is a specific introductory chapter for the cardiac work contained in Chapters 4, 5 and 6. Chapter 7 contains its own introduction relating to cartilage differentiation of stem cells, and this is followed by a final discussion chapter, Chapter 8, encompassing the whole thesis.

CHAPTER 2

Materials and Methods

2.1 Tissue Culture Plastic Coating

Tissue culture plates and flasks (Helena Biosciences, UK) were coated with bonding agents to improve cell adherence.

2.1.1 Fibronectin

Tissue culture flasks (Helena Biosciences, UK) were coated with a fibronectin/collagen coating comprising; 100 mL basal medium, 10 mL Bovine Serum Albumin (BSA) (1 mg/mL), 1 mL Collagen 1 (Bovine, 2.9mg/mL. BD, USA. Catalogue number; 40231), 1 mL Fibronectin (Human, 1 mg/mL. BD, USA. Catalogue number; 40008A). 100 µL per cm^2 was added to tissue culture flasks and incubated at 37 °C, 5% CO_2 overnight. Following this excessive coating was removed and flasks allowed to air dry in tissue culture hoods for 1 hour.

2.1.2 Poly-D-Lysine

Poly-D-Lysine (Sigma, UK) was diluted to 0.1 mg/mL using 1 x Phosphate Buffered Saline (PBS) (Gibco, UK). 100 µL was added to the base of each well of a 24 well plate (Costar, UK) and incubated at room temperature for 5 minutes. Poly-D-Lysine was discarded and each well was subsequently washed twice with PBS, following which the plates were wrapped in clingorap (Terinex, UK) and stored at -20 °C until usage.

2.1.3 Gelatin

Gelatin type A (Sigma, catalogue number G6144) was prepared as a 1% solution by adding 1 mg into 100 mL of PBS and autoclaved to produce a sterile solution. Gelatin was used at either a 1% or 0.1% dilution and was applied directly to the base of the flask or well on multi-well plates and incubated at 37 °C, 5% CO_2 for 15 minutes before discarding the gelatin solution. Flasks/plates could be used immediately or stored at 4 °C for 1 month.

2.2 Cell Counting

Harvested cultured cells were made up to a known volume using appropriate media. 10 µL of cell solution was added to an equal volume of trypan blue (Sigma, UK) and counted using a haemocytometer (Menzel-Glaser, Germany).

2.3 RNA Extraction, Cleanup and Quantification

2.3.1 RNA Extraction from Cell Pellets

Total RNA was isolated according to the protocol outlined in the RNeasy® Mini Kit (Qiagen, UK). Cell pellets totaling less than 5 x 10^6 cells were disrupted using 350 μL of Buffer RLT, and transferred to a Qiashredder tube (Qiagen, UK) and centrifuged at full speed for 2 minutes. 350 μL of 70% ethanol was mixed with the homogenized lysate and then transferred to a RNeasy spin column (Qiagen, UK) and centrifuged at >8,000g for 15 seconds. Flow through was discarded and 700 μL of Buffer RW1 was added to the column, and again centrifuged at >8,000g for 15 seconds. Flow through was discarded and 500 μL of Buffer RPE (containing ethanol) was added to the column and centrifuged at >8,000g for 15 seconds. Flow through was discarded and a second 500 μL of Buffer RPE was added to the column and centrifuged at >8,000g for 2 minutes. Flow through was discarded and the spin column was transferred to a new collection tube and centrifuged at >8,000g for 1 minute to remove residual ethanol. 50 μL of DEPC treated RNase free water (Ambion, UK) was added to the centre of the spin column membrane and incubated at room temperature for 10 minutes before being centrifuged at >8,000g for 1 minute to elute RNA. The RNA was subsequently cleaned up using the DNase 1 protocol (see below).

2.3.2 RNA Extraction from Tissue

Tissue sections harvested from recently euthanized animals were placed immediately into RNAlater (Ambion, UK) and stored at -20 °C. For RNA extraction, sections were subsequently dissected into 1-2mm^3 sections weighing approximately 5-10 mg and placed into lysing Matrix D tubes (MPBio, Germany) with 300 μL buffer RLT from the RNeasy® Fibrous Tissue Mini Kit (Qiagen, UK). The samples were then homogenized using FastPrep (Thermo Scientific, UK) for 40 seconds at level 6 and placed on ice for 10 minutes. Tissue lysate was then transferred (carefully leaving behind protein froth) into a new eppendorf and 590 μL of DEPC water added plus 10 μL of Proteinase K. The solution was mixed and placed at 55 °C for 10 minutes before being centrifuged at 10,000g for 3 minutes at room temperature. Supernatant was transferred to a new eppendorf leaving behind a debris pellet, and 0.5 volume of 100% ethanol was added and mixed. 700 μL of mixture was transferred to an RNeasy mini-column and centrifuged at >8,000g for 15 seconds. Flow through was discarded and remaining lysate mixture was added to the column and spun again. 700 μL of Buffer RLT was added to the column and the column centrifuged at >8,000g for 15 seconds. Flow through was discarded and 500 μL of Buffer RPE was added to the column and centrifuged at 8,000g for 15 seconds and flow through discarded again. A further 500 μL of Buffer RPE was added to the column, and the column centrifuged at 8,000g for 2 minutes. The column was transferred to a new collection tube and centrifuged for 1 minute to evaporate excess ethanol. 50 μL of DEPC treated water was added to the column and subsequently

centrifuged at >8,000g for 1 minute to elute the RNA. The RNA was subsequently cleaned up using DNase 1 protocol (see below).

2.3.3 RNA Cleanup

RNA was DNAse treated to eliminate genomic DNA contamination using the protocol outlined in the RNase-Free DNase Set (Qiagen, UK). RNA solution was made up to 87.5 μL using DEPC treated water, and 10 μL of Buffer RDD and 2.5 μL of DNase 1 stock solution were added. The mixture was incubated at room temperature for 10 minutes before commencing RNA cleanup. 350 μL of Buffer RLT plus 250 μL of 100% ethanol was added to the solution and transferred to a spin column and centrifuged at >8,000g for 15 seconds. Flow through was discarded and 500 μL of Buffer RPE was added and the tube centrifuged at >8,000g for 15 seconds. Flow through was discarded and a further 500 μL of Buffer RPE was added before being centrifuged at >8,000g for 2 minutes. The spin columns were then transferred to a new collection tube and centrifuged at >8,000g for 1 minute to evaporate ethanol. 35 μL of DEPC treated water was placed in the centre of the column membrane and incubated at room temperature for 10 minutes before a final centrifuge spin at >8,000g for 1 minute to elute RNA.

2.3.4 Total RNA Quantification

Eluted RNA was quantified and quality checked using 260:280 nm absorption ratios on the Thermo Scientific Nanodrop ™ machine (Thermo Scientific, USA).

2.4 Semi-Quantitative RT-PCR

2.4.1 Primer Design

Primer pairs for canine and murine markers were designed based on published genome sequences (NCBI) and the Primer3 software (http://primer3.sourceforge.net/). Individual primers are displayed in the chapters of relevance. All primers where possible were designed as intron spanning. In the case of canine primers they were designed to allow for multiplexing with GAPDH primers. Nkx2.5 primers were taken from Vaags *et al* (Vaags et al., 2009) and GADPH primer sequences were used as a housekeeping control gene and were generously donated by Dr. Karen Tan.

2.4.2 Primer Preparation

All primers were ordered through MWG Eurofins, and were HPSF quality. Upon arrival primers were diluted to 100 pmol/μL and stored at -20 °C. Melting temperatures for each primer pair were optimized upon arrival using RT-PCR (see below).

2.4.3 Reverse Transcription

Extracted RNA was reverse transcribed using the protocol outlined in the Omniscript® Reverse Transcription kit (Qiagen, UK) using Random Nanomers (Sigma-Aldrich,

USA) at an optimised final concentration of 2.5 µmol/L, and an RNase inhibitor (Promega, UK. Catalogue number N2511). A Reverse Transcription (RT) master mix was prepared as described in Table 2.1.

Table 2.1. Reverse transcription master mix components and volumes.

Master Mix Component	***1 x (µL)***
10 x Buffer	2
RNase inhibitor	0.25
dNTP	2
DTT	2
Random Primers	2
Omniscript RT Enzyme	1

Total RNA was made up to a final volume of 10.75 µL using DEPC treated water and incubated at 65 °C for 5 minutes, before chilling on ice. 9.25 µL of RT master mix was then added to the RNA and incubated at 40 °C for 1 hour before storing newly formed cDNA at -20 °C. For semi-quantitative analysis all samples had the same total RNA input at RT step.

2.4.4 Polymerase Chain Reaction

Polymerase chain reaction (PCR) master mixes were prepared for all canine experiments (murine experiments are outlined in Chapter 6) using the Promega Go-Taq PCR kit (Table 2.2).

Table 2.2. PCR master mix components and volumes.

Master Mix Component	*1 x (µL)*
DEPC Treated Water	32.05
dNTP	1
$MgCl_2$	4
5 x GoTaq Buffer	10
GAPDH Forward Primer	0.1
GAPDH Reverse Primer	0.1
Target Forward Primer	0.25
Target Reverse Primer	0.25
GoTaq polymerase	0.25

48 µL of PCR master mix was added to 2 µL of sample cDNA in a PCR reaction tube (Axygen, USA). PCR cycles were run using the Bio-rad iCycler. The following protocol was found to be optimal for the reactions: An initial denaturation step of 95 °C for

5 minutes. This was followed by 35 cycles of denaturation at 95 °C for 1 minute, annealing at primer specific temperatures for 1 minute and extension at 72 °C for 1 minute. The program was completed with a final extension step of 72 °C for 10 minutes. Samples were stored at 4 °C until analysis. Gene target primers were run at a final concentration of 0.5 pmol/μL with GAPDH multiplexed within the reaction at a final primer concentration of 0.2 pmol/μL, thus creating an internal positive control. Negative controls of RNA and water were run alongside to confirm absence of genomic DNA contamination.

2.4.5 Agarose Gel Preparation and PCR Product Analysis

PCR products were run on a 2% agarose gel. This was prepared by adding 2 g of agarose (Sigma, UK) with 100 mL of 1 x Tris Acetate EDTA (TAE) (Gibco, UK) and melted using a microwave. 10 μL GelRed 10,000X (Biotium) was added to the 100 mL melted agarose and the agarose was poured into a setting tray with inserted comb and allowed to set at room temperature. 6 μL of PCR product was added to 2 μL of 6 X Blue/Orange Loading Dye (Promega, UK) and mixed before loading into the gel. A 100 base pair (bp) ladder (Promega, UK) was run as a size control alongside samples. The samples were run though the gel using electrical current (Bio-Rad, UK) to create band separation and visualised using the BioRad Molecular Imager GelDoc system.

2.4.6 Gel Band Excision and Cleanup

PCR products were run on a 2% agarose gel as described above, and the bands visualised using a UV transilluminator. Bands at the expected product size were excised from the gel and DNA extracted and purified using the Wizard® SV Gel and PCR Clean-up System (Promega, USA). Purified DNA products were stored at -20 °C.

2.4.7 PCR Product Sequencing

PCR products were extracted as described previously. DNA concentration was determined using the Thermo Scientific Nanodrop ™ (Thermo Scientific, USA). Sequencing reactions were prepared using 3-10 ng of PCR product in 5 μL RNAse free H_20 (Ambion, UK), and 3.2 pmol of either forward or reverse primer in 1 μL of RNAse free water. Reactions were sequenced with BigDye®Terminator kit (Applied Biosystems, UK) and run on the ABI 3730 DNA Analyzer (Applied Biosystems, UK). Sequencing was analysed using the Sequence Scanner v1.0 (Applied Biosystems, UK) and compared to the canine genome (NCBI).

2.5 Quantitative RT-PCR

2.5.1 Primer Design

Primers for target genes and probes were designed using the LightCycler® Probe Design Software 2.0 (Roche). Where possible intron spanning primers were selected. A Eukaryotic 18s rRNA endogenous control VIC/MGB probe and primer were utilised as housekeeping internal control primers (Applied Biosystems, UK. Catalogue number: 4319413E).

2.5.2 Primer and Probe Preparation

Primers were ordered from MWG Eurofins (HPLC purified). Individual FAM probes were ordered from Roche Applied Science, UK. Upon arrival primers were diluted to 50 μmol/L (50 pmol/μL) using DEPC treated water. A 10 X primer/probe stock solution was prepared by adding 1 part forward primer, 1 part reverse primer, 1-part probe and 7-parts water. This was stored at -20 °C and protected from light.

2.5.3 Reverse Transcription

RNA was extracted and cleaned up as previously described. cDNA was generated using the Roche Hi-Fidelity cDNA synthesis kit (Roche, UK). A master mix was prepared as desribed in Table 2.3.

Table 2.3. Reverse transcription master mix for cDNA for quantitative RT-PCR reactions.

Master Mix Component	*1 x (µL)*
Buffer (5x)	4
RNAse inhibitor	0.5
dNTP	2
DTT	1
RT enzyme	1.1

Total RNA was made up to a final volume of 9.4 µL using DEPC treated water and 2 µL of random primers was added to this. This was heated to 65 °C using the Bio-rad iCycler and then allowed to cool before adding 8.4 µL of master mix to each reaction. The samples were then transferred to the Bio-rad iCycler for the cycle programme described in Table 2.4.

Table 2.4. Programme settings for reverse transcription step for quantitative RT-PCR.

Temperature °C	*Time (mins)*
29	10
48	60
85	5
8	ω

Samples were then pooled and aliquots (if large volumes were prepared) stored at -20 °C.

Individual quantitative RT-PCR reactions are described in the relevant chapters. Data processing was performed using Microsoft® Excel.

2.6 Western Blot Analysis

2.6.1 Cell Harvesting and Cell Lysis

Cells were harvested from culture using 0.05% trypsin/EDTA (Invitrogen, UK) and pelleted by centrifugation at 1200 rpm for 5 minutes at room temperature. Supernatant was discarded and pellets re-suspended in 1 mL of PBS. Cell suspension was again centrifuged at 1200 rpm for 5 minutes and supernatant discarded. Pellets were disrupted using 2 x cell pellet volume of NP40 lysis buffer (1% NP40; 150mM KCl; 25mM pH 7.4 Hepes; 5mM DL-Dithiothreitol (DTT); 50mM NaF and 1x Protease Inhibitor Mix (all Sigma, UK)) on ice for 15 minutes. The mix was centrifuged at 14000 rpm for 15 minutes at 4 °C and the lysate was extracted and snap frozen before storing at -80 °C.

2.6.2 Bradford Assay

Bradford standards were prepared by dilution of neat Bradford stock (10mg/mL) (Bio-Rad, UK) in a 2:3 ratio with water creating a 4 mg/mL stock, followed by 5 serial dilutions through to a 0.125 mg/mL solution. 5 μL of each standard was added to 200 μL of Bradford reagent (Bio-Rad, UK) in duplicate across a white 96 well plate (Corning, UK). 2 μL of sample protein lysate was added to 200 μL of Bradford reagent in a separate well and the absorbance was recorded using a platemeter (Victor3, Perkin Elmer, USA) with a standard curve with R^2 value plotted using the Bradford standard data. The absorbance of the protein lysate of interest was plotted against the curve indicating mg/mL of the extracted protein.

2.6.3 SDS Polyacrylamide Gel Electrophoresis

Quantification of protein concentration was performed using Bradford Assays and proteins were resolved on the basis of their molecular weight using SDS polyacrylamide gel electrophoresis (SDS PAGE). Proteins resolved by the SDS PAGE were transferred to a nitrocellulose membrane (Amersham™ Hybond™, GE Healthcare UK) and blocked using 5% skimmed milk (Fluka, Switzerland) in 0.1% PBS/Tween (0.1% Tween in PBS). Blot probing is described in further detail in Chapter 4; Cardiac Stem Cells.

2.7 Immunofluorescence Analysis

Cells were grown on 4 well chambered slides (Corning, UK) under cell specific conditions until slides were approximately 50% confluent. Medium was aspirated and slides washed in PBS, which was subsequently discarded. Slides were briefly air dried and then fixed directly in 4% paraformaldehyde for 20 minutes, followed by two PBS washes and subsequently placed in 100% ethanol for 5 minutes, followed by two PBS washes to permeabilise the cells. Blocking buffer (10% normal goat serum (Invitrogen, UK); 0.1% Tween 20 (Sigma UK), in 1x PBS) was applied for 1 hour at room temperature, and slides were incubated with primary antibody of interest, diluted in PBST (0.1% normal goat serum; 0.1% Tween 20 in PBS) and incubated overnight in a humidified chamber at 4 °C. Following this, two washes with PBS for 5 minutes were performed, followed by incubation with the secondary antibody of choice, for 1 hour at room temperature in a dark humid chamber. Nuclear counterstaining was performed using DAPI mount (Vectastain) after three 5 minute washes in PBS. Coverslips were placed over the mount, and edges sealed using nail varnish. Images were taken using the fluorescent microscope (Zeiss, Axiovert 40) and analysed using the Zeiss Axiovision 4.7 software. Slides were stored at -20 °C. Specific details of immunofluorescence can be found in individual results chapters.

2.8 Cardiac Directed Differentiation using Co-Culture

Indirect co-culture was performed to allow analysis of both mesenchymal stem cells (MSCs) and cardiac stem cells (CSCs) whilst in co-culture with an autologous source of stem cells. The general experimental procedure was similar for both cell populations and is described below. Individual results for CSCs and MSCs can be found in Chapters 4 and 5 respectively.

2.8.1 Isolation and Culture of Cardiac Stem Cells and Mesenchymal Stem Cells

Cardiac explants and bone marrow were taken from dogs recently euthanized by pentobarbital overdose. CSCs and MSCs were cultured from these tissues and grown concurrently to produce viable stem cells of each source. Individual detail of CSC and MSC isolation and culture can be found in materials and methods of Chapters 4 and 5 respectively.

2.8.2 Harvest of Cardiac Stem Cells and Mesenchymal Stem Cells

At approximately 4 weeks after harvesting explants both CSCs and MSCs were ready for collection. Detailed description of cellular harvests for CSCs and MSCs can be found in the materials and methods of Chapters 4 and 5 respectively, however briefly;

1. Cardiac Stem Cells: Media from explant flasks was pooled to collect all free floating phase bright cells. Flasks were then gently rinsed using PBS and this added to the media to pool cells. Cell suspension was centrifuged at 1200 rpm for 5 minutes at room temperature to pellet cells. Cells were re-suspended in PBS and counted as previously described.

2. Mesenchymal stem cells: MSC media was removed from the flasks and discarded and the cells washed in PBS. 0.05% Trypsin/EDTA was added to the flasks and the flasks subsequently incubated at 37 °C, 5% CO_2 for 10 minutes to loosen cells. 10 mL of MSC media was added to the flasks and the cell suspension removed. Cells were centrifuged at 1200 rpm for 5 minutes at room temperature to pellet the cells, and then re-suspended in PBS and counted.

Both CSCs and MSCs were then re-centrifuged at 1200 rpm for 5 minutes at room temperature before being re-suspended in co-culture Differentiation Enabling Media (DEM); DMEM (high glucose, with L-Glutamine, Catalogue number 41965, Invitrogen UK), 10% foetal bovine serum (FBS), 10% ITS+ premix (BD Biosciences, UK), 10% Na Pyruvate (Gibco, UK), 10% MEM-NEA (Gibco, UK), 1 x Ascorbic Acid (Sigma, UK), 0.1 mmol/L 2-Mercaptoethanol (2-ME), 100 U/mL Penicillin G and 100 μg/mL Streptomycin (both Invitrogen, UK).

2.8.3 Plate Set-up for Co-Culture Experiment

A plate plan for differentiation is shown is shown in Figure 2.1:

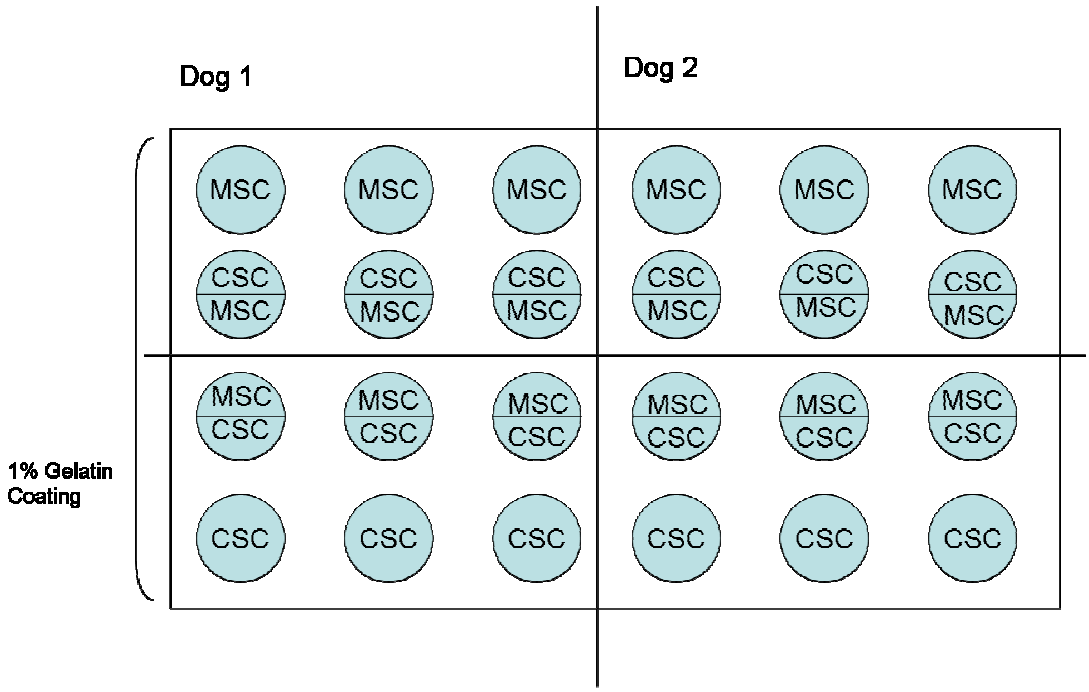


Figure 2.1. Plate plan for CSC and MSC indirect co-culture. One 24 well plate could be used for one timepoint, including two dogs. On each plate were control wells of both cell sources cultured alone, and co-culture wells of both CSCs and MSCs with the alternative cell source grown in transwell inserts above. All wells were coated with 1% gelatin prior to initiation.

Collagen-Coated Transwell® - COL Inserts, 0.4 μm diameter were purchased from Corning Lifesciences, USA (Catalogue number; 3495). A single 24 well plate containing

12 Collagen transwell inserts was used for each time point (day 6 and day 10), with two dogs on one plate. The base of each well was coated with 1% gelatin to improve cell binding. Four individual permutations of experiment were run, each with triplicate wells;

1. Mesenchymal stem cells alone
2. Mesenchymal stem cells at the base of the well, with cardiac stem cells grown on a transwell insert above
3. Cardiac stem cells at the base of the well, with mesenchymal stem cells grown on a transwell insert above
4. Cardiac stem cells alone

2 x 10^4 cells were seeded into base wells of the 24 well plate (growth area 1.9 cm^2) in 600 μL of DEM media, with 1 x 10^4 cells seeded onto transwell inserts (growth area 0.33 cm^2) in 100 μL DEM media. Plates were then placed at 37 °C, 5% CO_2.

Following plating, remaining cells were re-centrifuged at 400g, for 10 minutes at 4 °C to pellet cells. Cells were then washed in chilled PBS and re-centrifuged as above. The PBS was discarded and the cell pellet snap frozen and stored at -80 °C as day 0 control cells.

Medium was refreshed at days 3 and 8 post initiation. In the base wells 300 μL of DEM media was carefully removed and 400 μL of fresh DEM media replaced and from the transwell inserts 50 μL of spent media was carefully removed and 80 μL of fresh DEM medium was replaced.

2.8.4 Time Point Harvest

Triplicate wells were harvested from each plate at two time points, day 6 and 10. Transwell inserts were removed and discarded and images were taken of the cell monolayer. Medium was removed and the cells washed in PBS. 0.05%. Trypsin/EDTA was added to each well and the plate placed at 37 °C, 5% CO_2 for 5-10 minutes. Following this 1 mL of warm DEM media was added to each well and the cell suspension transferred to a 15 mL Falcon tube. The cells were then centrifuged for 10 minutes at 400g at 4 °C to form a pellet. Media was removed and discarded and 1 mL of chilled PBS was added and the cells washed before being centrifuged again at the same settings to re-pellet the cells. PBS was discarded and the pellet snap frozen, before storing at -80 °C.

2.8.5 Analysis

Cell pellets were removed from -80 °C storage and thawed. RNA was extracted from each pellet using the RNeasy Mini Kit and cleaned up using the DNase 1 Kit as described previously. RNA was quantified using the Nanodrop machine to calculate total RNA and quality. At this point RNA from each triplicate was pooled into one combined sample which was again quantified using the Nanodrop machine to calculate a combined total RNA. This RNA was used for reverse transcription, using 150 ng of total RNA (except for cardiac stem cells at day 6, where 50 ng was used due to low total

RNA) and the Omniscript RT kit as described previously. cDNA was stored at -20 °C until use.

Semi-quantitative RT-PCR was performed using the prepared cDNA and all canine primers (see individual chapters), analysing day 0, 6 and 10 time points for both co-cultured cardiac and mesenchymal stem cells and the stem cell only controls.

2.9 Image Capture and Processing

Cell morphology images and immunofluorescence were viewed using the Zeiss Axiovert 40 CPL microscope. Images were processed using the image software Axiovision 4.7.2 (12-2008).

CHAPTER 3

Heart Disease and the Need for Stem Cell Research

3.1 Cardiac Disease and the Dog

In pet pedigree dogs cardiac disease is the third biggest cause of mortality after cancer and 'old age' and is therefore of significant importance in veterinary practice. Cardiac diseases in dogs are predominantly seen in two major categories; cardiomyopathies and valvular degeneration, with congenital causes of disease generally very rare (Egenvall et al., 2006, Adams et al., 2010). Risk of death from cardiac disease increases with age of patient, and is also highly breed specific. For example, large breed dogs, such as Great Danes and Irish Wolfhounds are at major risk of developing dilated cardiomyopathy (DCM) whereas the Cavalier King Charles Spaniel has a significant risk of developing myxomatous mitral valve disease (MVMD) (Egenvall et al., 2006). Managing cardiac disease morbidity in the dog has been the focus of significant pharmaceutical research, with the key goal being the improvement of quality of life first and foremost, followed by increased longevity. Treatment protocols have been released for both DCM and MVMD which alleviate some of the symptoms of heart failure, however no treatment regime will halt disease progression (Borgarelli et al., 2001, Tidholm et al., 2001,

Haggstrom et al., 2009). The need for the study of cardiac diseases in the dog is therefore evident, and the use of stem cells for this is an exciting prospect, not only for study of disease processes *in vitro*, but also for their future therapeutic potential *in vivo.*

Cardiac disease is the number one killer of the human population globally and in 2005 approximately 17.5 million people died from cardiovascular disease, representing 30% of all deaths worldwide. Many similarities can be drawn between humans and dogs, and through the study of cardiac disease in both species we may generate beneficial information which can be transferred across to human medicine and vice versa, thus allowing for the dog to become a model for human disease.

3.1.1 The Genetic Influence in Cardiac Disease

Genetic causes of cardiac disease are an important area of study in order to allow for future limitation of inherited traits contributing to the development of disease. It is known that there is a strong genetic influence in canine cardiac disease; this is highlighted in pedigree dogs, where specific conditions are more common in certain breeds. As already mentioned, MVMD is most common in Cavalier King Charles Spaniels with high heritability, and the likelihood of polygenic traits influencing the disease (Lewis et al., 2010). Boxers are predisposed to arrythmogenic right ventricular cardiomyopathy (ARVC) (Oyama et al., 2008), and Doberman Pinschers to DCM (Wess et al., 2010). Even though the evidence for genetic association is very strong in pedigree dogs no specific genetic mutations have yet been found.

Study of genetic influences of cardiac disease may be enabled by the isolation and culture of stem cells from affected patients, allowing for disease study *in vitro* on a cell population carrying the genetic flaw (Beqqali et al., 2009). In human cardiovascular disease many of the genetic mutations have already been characterised and rodent models created (Figure 3.1). These models, although useful, are not an ideal direct comparison and therefore studying cell populations from pedigree dogs affected by each individual condition would enable a more direct and relevant analysis for veterinary medicine than rodent models.

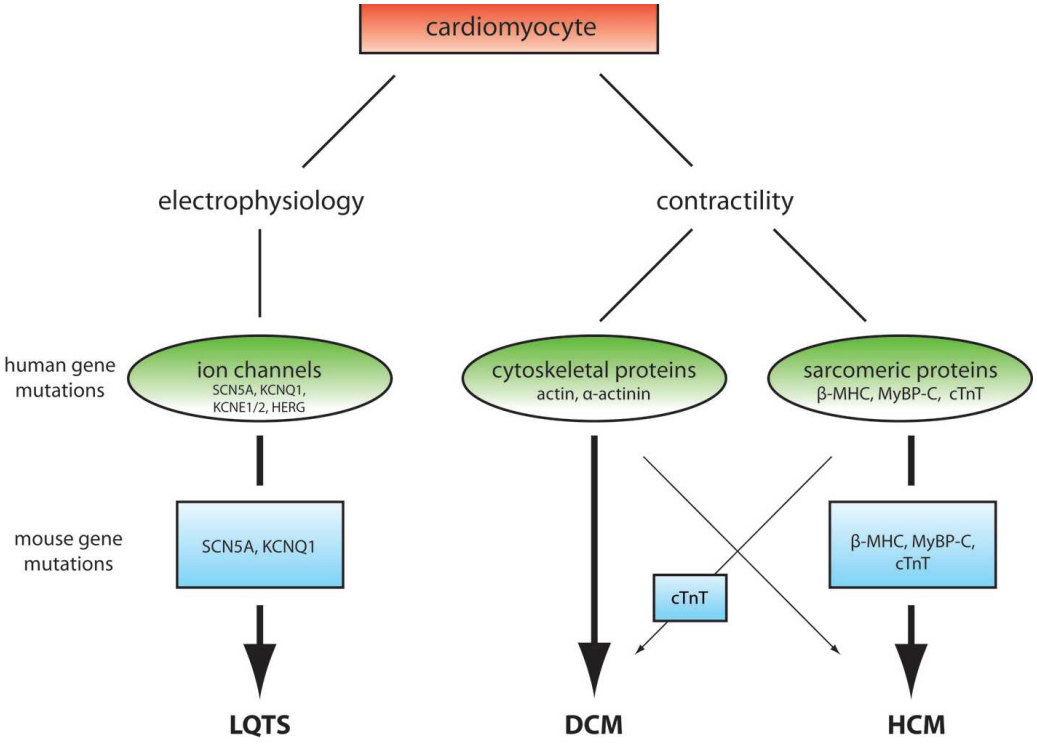


Figure 3.1. Human genetic mutations causing cardiovascular disease. Mutations in ion channel and cellular proteins lead to diseases of channels or contractility (channelopathies and cardiomyopathies respectively). Green represents the known genetic mutation found in the human, with blue the corresponding mouse model. LQTS-Long QT Syndrome; DCM-Dilated Cardiomyopathy; HCM-Hypertrophic Cardiomyopathy; SCN5A-Sodium Channel, Voltage-gated, type V, Alpha subunit; KCNQ1-Potassium Voltage-gated Channel, KQT-like Subfamily, Member 1; KCNE1/2-Potassium Voltage-gated Channel, Isk-related family, Member 1/2; HERG-Potassium Voltage-gated Channel, Subfamily H (eag-related), Member 2 ; CTT-Cardiac Troponin T; β-MHC-β-Myosin Heavy Chain; MyBP-C-Myosin Binding Protein-C. Taken from (Beqqali et al., 2009).

The study of genetically flawed cardiomyocytes derived from stem cells would allow for new therapeutics to be tested on the cultured cell populations, giving an initial idea of the affect these drugs may have on the patients. Furthermore, the use of stem cells in this way *in vitro* may act as an animal sparing source of testing material in pharmacological research. Cardiomyocytes derived from ES cells have been found to demonstrate similar responses to cardiac and non-cardiac drugs *in vitro* as have been seen *in vivo*, thus highlighting the potential of stem cells for future drug development (Braam et al., 2010). The use of stem cells therapeutically is also being investigated, with some positive effects seen with the direct application of stem cells, predominantly MSCs, into damaged hearts (this is discussed in more detail in Chapter 5).

3.2 The Heart is Not a Post-Mitotic Organ

The heart was long thought to be a terminally differentiated organ with no capacity for renewal and repair. Interestingly the Zebrafish is capable of cardiac repair and is a model for cardiac damage due to its ability to fully regenerate the damaged heart within 2 months after a 20% ventricular resection (Poss et al., 2002, Lepilina et al., 2006). The process by which this happens is a matter of great interest as it may elucidate areas for research in mammalian models. In humans recent evidence has shown that the heart does have regenerative capacity, albeit at low levels. Using Ki-67 staining and genetic fate mapping it has been shown that regeneration in the heart is increased following injury, and therefore suggests that cardiac renewal is possible, but not part of the normal

ageing process (Beltrami et al., 2001, Hsieh et al., 2007). In contrast it has been shown using carbon-14 dating that renewal is constantly occurring, and is at its highest level in humans under 25, and decreases to under half the original level by the age of 75 (Bergmann et al., 2009). Furthermore, it has now been shown that females have a superior ability for cardiomyocyte renewal than males, with myocyte replacement occurring 15 times in a lifetime in a female compared with just 11 in a male (Kajstura et al., 2010). It would be fair to extrapolate from these results and assume that the dog also has a level of cardiomyocyte turnover, and that the presence of stem cells within the heart may contribute to normal remodelling and repair.

3.2.1 Difficulty in Culturing Primary Cardiomyocytes

Stem cells have offered an option to study cardiac diseases *in vitro* which until recently had been made challenging by the difficulty in culturing primary cardiomyocytes. Yield is notoriously low, and long-term subculture is very difficult and leads to phenotypic changes, including loss of sarcomeres by 10 days in culture and loss of myofilaments with increasing passage (Li et al., 1996, Bird et al., 2003). This has meant that use of primary cardiomyocytes *in vitro* was severely limiting and unreliable for direct translation *in vivo* and therefore stem cells have become a promising alternative option.

3.3 Differentiation of Stem Cells to Cardiomyocytes

3.3.1 Derivation of Cardiomyocytes from Embryonic Stem Cells

ES cells have been used to derive cardiomyocytes which can then be used for functional analysis, pharmacological studies, and also for transplant studies. Crucially the signalling pathways and the culturing conditions between undifferentiated and differentiating ES cells are not identical between mice (mES) and humans (hES). Spontaneous differentiation of ES cells towards a specific lineage is poor, specifically for hES cells (Puceat, 2008). The conventional approach to induce cardiac differentiation was devised using mES cells and involved the formation of embryoid bodies, which were then 'hung' from tissue culture plates, and from which differentiated cells derived from endoderm, mesoderm and ectoderm were isolated (Desbaillets et al., 2000). Subsequent plating of the mesodermal cells resulted in areas of spontaneous contraction appearing within 1-4 days for mES cells (Boheler et al., 2002, Sachinidis et al., 2003). An increase in the percentage of cells differentiating toward the cardiac lineage can be achieved by exposing the ES cells to bone morphogenic protein 2 (BMP 2) prior to the formation of hanging drops (Puceat, 2008) or BMP 2 and TGFβ in combination (Behfar et al., 2002); alternatively up-regulation of cardiogenic genes can be seen in embryoid bodies treated with the reprogramming cytokine tumour necrosis factor-α (TNF-α) initially added to try to limit neoplastic growth (Behfar et al., 2007).

hES derived mesodermal cells isolated from the embryoid body can also differentiate into cardiomyocytes, producing areas of spontaneous beating, but require the presence of serum. Directed differentiation can be further promoted toward the cardiac lineage with combinations of growth factors. An efficient system utilising Activin A and bone morphogenic protein 4 (BMP 4) has been described, which results in a 71-95% conversion of mesodermal ES cells to beating cardiomyocytes (Laflamme et al., 2007). Alternatively co-culture of hES cells with the murine endodermal cell line, END-2 in the absence of serum also creates spontaneously beating cardiomyocytes (Mummery et al., 2003, Passier et al., 2005).

Canine ES cells have only been described in the last 5 years, and are therefore much further behind in terms of research than human and mouse ES cells. However, canine ES cells are capable of differentiating along all three germ layers; mesoderm, endoderm and ectoderm (Hayes et al., 2008, Vaags et al., 2009). More specific differentiation has been found toward neuronal cells and haematopoietic stem cells (Schneider et al., 2007, Wilcox et al., 2011). Only two references to specific cardiac differentiation currently exist. Hatoya *et al* demonstrated the most successful cardiac differentiation using the embryoid body technique, and found beating cells after 1-2 weeks (Hatoya et al., 2006) whereas Vaags *et al* analysed gene expression following a similar differentiation technique which demonstrated a slight increase in cardiac specific markers GATA 4 and myosin light chain 2v (Vaags et al., 2009).

3.3.2 Derivation of Cardiomyocytes from Adult Stem Cells

The heart had long been thought of as a terminally differentiated organ, with a limited capacity for repair. However, the discovery of adult stem cells instigated the search for a population of progenitor cells specific to the heart. Following work demonstrating mitosis and cytokinesis in human hearts under normal conditions it was found that the heart did indeed have a natural population of stem-cell like cells (Anversa and Kajstura, 1998, Beltrami et al., 2001, Nadal-Ginard et al., 2003) Messina *et al* identified and characterised a cell population from both the mouse and human heart with properties of CSCs. The isolated cells grew as self-adherent clusters, which they termed 'cardiospheres'. These cells were capable of both self-renewal and could differentiate *in vitro* and *in vivo* into the major specialised cell types of the heart; cardiomyocytes, endothelial cells and smooth muscle cells (Messina et al., 2004). Stem cells derived from the bone marrow have also been driven toward cardiac lineages *in vitro* using various techniques, and may present a more accessible source of stem cells for cardiac differentiation than CSCs (Makino et al., 1999, Fukuda, 2001). More detailed descriptions of cardiac differentiation from adult stem cells are found in individual chapters.

3.3.3 Derivation of Cardiomyocytes from Induced Pluripotent Stem Cells

The development of iPS cells has taken stem cell biology into a new era, with the possibility of creating patient specific treatments. Mauritz *et al* and Narazaki *et al* both

described the production of functional murine cardiomyocytes from iPS cells – the differentiated cells displayed functional characteristics similar to that of ES cell derived cardiomyocytes. Mauritz *et al* generated murine iPS cells containing all four iPS transcripts (Oct 4, c-Myc, KLF4 and SOX2), which expressed high levels of endogenous Oct 4, Nanog and SSEA 1. These were cultured initially on MEF feeders in the presence of LIF before generating embryoid bodies which were subsequently plated. The iPS cells began spontaneously contracting from around day 12 of plating, and the beating cells were found to be expressing markers for cardiomyocyte lineages including GATA 4, Nkx2.5 and those for differentiated cardiac cells including α-myosin heavy chain, cardiac troponin T and connexin 43. The beating cells were analysed for their functionality using microelectrode arrays for electrical impulses, and intracellular calcium measurements using confocal laser microscopy, and were found to be behaving as functional cardiomyocytes (Mauritz et al., 2008). Nazaraki *et al* generated murine iPS cells including all four transcripts, and then induced a population of mesodermal cells expressing Flk-1 (vascular endothelial growth factor receptor-2). The purified Flk-1 population was capable of differentiation into endothelial cells and cardiomyocytes (Narazaki et al., 2008). Zhang *et al* generated a human iPS cell line using Oct 4, Nanog, SOX2 and Lin 28 transgenes, without the requirement for the oncogenic c-Myc gene. They then differentiated these cells using the embryoid body method and created functional cardiomyocytes, which were responsive to β-adrenergic stimulation shown by an increase in spontaneous contraction rate and a decrease in action potential duration (Zhang et al., 2009). Moretti *et al* found using genetic fate mapping, that iPS cells positive for islet 1 were capable of spontaneous differentiation along the three main

cardiac routes in both mice and humans without teratoma formation (Moretti et al., 2010). Following on from this work, murine chimeras have been generated from iPS cells derived with and without the presence of c-Myc, using human genes, which were capable of forming functional normally developed hearts (Martinez-Fernandez et al., 2009, Nelson et al., 2009). Most recently it has been found that the addition of small molecule Wnt inhibitors can increase the efficiency of cardiac differentiation of human iPS cells (Ren et al., 2011). In all differentiation experiments the iPS cells were compared to an ES cell line from the same species, and behaviour in culture and differentiation patterns were found to be similar between the two.

Canine iPS cells have only recently been described, but have been found to be capable of differentiation along all three germ lines using embryoid body techniques (Shimada et al., 2010, Lee et al., 2011, Luo et al., 2011). This data compares favourably with that produced for human and mouse iPS cells and will likely pave the way for future research into more specific differentiated tissues such as cardiomyocytes.

3.3.3.1 Epigenetic Memory and Direct Re-programming

iPS cells have traditionally been created using fibroblasts as the original somatic cell used for reprogramming. However, early stage research has suggested that iPS cells retain some epigenetic memory of their tissue of origin. For example, mouse iPS cells created using early bone marrow lineage cells were found to produce higher numbers of haematopoietic colony forming cells compared to using fibroblast cells as the

transformed cell (Kim et al., 2010). Furthermore it has been found that by altering the traditional genes inserted into the genome to those which are more tissue specific it is possible to directly re-programme somatic cells to a new tissue whilst by-passing the pluripotent stage. Vierbuchen *et al* have found that the insertion of three factors, Ascl1, Brn2 and Myt1l, could directly reprogramme adult fibroblasts into functional neurons *in vitro* (Vierbuchen et al., 2010). Excitingly, it has also been found that adult murine fibroblasts could be directly re-programmed to the cardiac lineage by the insertion of three key developmental factors; GATA 4, MEF2C and Tbx5. These reprogrammed cells demonstrated marker expression similar to cardiomyocytes and contracted spontaneously. Furthermore, insertion of the three factors directly into cardiac fibroblasts increased transcriptional activity compared to fibroblast derived cardiomyocytes, suggesting a combined epigenetic memory influence (Ieda et al., 2010).

3.3.4 Isolating Cardiac Precursors

A recent study has found that it is possible to isolate and select for cardiomyocytes derived from ES cells and iPS cells from within the mixed population of cells post-differentiation based upon high mitochondria content. Importantly, these cells were not capable of forming teratomas, and thus this technique may increase the ability to select for cardiomyogenic cells more specifically, and increase safety of using them *in vivo* (Hattori et al., 2010).

3.4 Stem Cell Derived Cardiomyocytes in Disease Models and Therapy

3.4.1 Embryonic Stem Cells and Cardiac Disease

Transplant studies have shown the improved repair of infarcted tissue following injection of stem cell derived cardiomyocytes. Laflamme *et al* found that hES cell-derived cardiomyocytes injected into infarcted immunodeficient rat hearts formed myocardial grafts in the presence of a cocktail of pro-survival factors, which attenuated ventricular dilation and preserved regional and global contractile function compared to controls receiving non-cardiac hES cell derivatives (Laflamme et al., 2007). Van Laake *et al* also found that injection of hES cell-derived cardiomyocytes into both healthy and myocardial infarcted murine hearts formed a graft, and in the case of the infarcted hearts, initially caused a significant improvement in cardiac function measured by magnetic resonance imaging (MRI). However, the improvement was not sustained at 12 weeks, although the graft was still present (van Laake et al., 2007). In many of the hES cell cardiomyocyte studies there appeared to be a transient improvement in cardiac function, but this was not sustainable long term; increased numbers of injected hES cell cardiomyocyte derived cells did not increase longevity of increased function, but did produce larger grafts within the myocardium. It was noted that over a ten week period gap junctions appeared to form within the graft, however connections from the graft to the host myocardium were rare. Often the grafts were surrounded by an extracellular matrix, although this did not preclude any electrical coupling. Given that mouse and human heart rates vary considerably (mouse 400-600 bpm, human 60-100 bpm) it may

be that electrical coupling was physiologically impossible. No arrhythmias were found in the host hearts, but until this system is trialed in larger animal models, which have slower heart rates, it cannot be assumed that none would occur (van Laake et al., 2008) .

Canine ES cells are still in their early phase of research and therefore have not yet been utilized for the treatment of cardiovascular diseases. Canine ES cells are challenging to produce, and as yet have not fulfilled all the criteria of an ES cell, such as teratoma formation and therefore the basic science of these cells will need to be investigated further before they can be considered for therapeutic use.

In general, concerns regarding the use of ES cell derived transplants remain, including the ethical issues surrounding the production of ES cells, the production of teratomas, and also an immunogenic response to engrafted cells within same species, and even genetically identical subjects (Swijnenburg et al., 2005).

3.4.2 Adult Stem Cells and Cardiac Disease

Two main sources of ASCs have been investigated for the treatment of cardiac disease. Firstly cardiac stem cells (CSCs), found in the heart itself. Implantation of adult cardiac stem cells (CSCs) has been found to improve cardiac function following myocardial infarction in mice (Smith et al., 2007, Matsuura et al., 2009), and in rats following implantation of human adult c-Kit positive CSCs (Beltrami et al., 2003). Secondly MSCs, located both in the bone marrow and adipose tissue have been used to treat

cardiac disease (Silva et al., 2005, Plotnikov et al., 2007). These cell sources are the main focus of this study and shall be described in more detail throughout the specific chapters. Alternative sources for cellular therapy have been described with limited success including primary foetal cardiomyocytes (Sakai et al., 1999) and skeletal myocytes (Jain et al., 2001, Menasche et al., 2001, Ghostine et al., 2002).

3.4.3 Induced Pluripotent Stem Cells and Cardiac Disease

The injection of murine iPS cells intramyocardially into immunodeficient mice generated the growth of teratomas, as would normally be expected of iPS cells. However, when injected subcutaneously and intramyocardially into immunocompetent mice no teratomas formed, and furthermore the iPS cells stably engrafted into the heart itself (Nelson et al., 2009). This may indicate that in future, patient specific iPS cells may be used to target damaged myocardium.

Cardioactive therapeutics have been tested on cardiomyocytes derived from human iPS cells. The iPS cells were found to respond to the cardiac drugs similarly to both ES cell derived cardiomyocytes and human cardiomyocytes. The use of iPS cells in this field would allow not only for cardiac drug screening *in vitro* but more importantly would allow for patient specific testing, individualizing and tailoring drugs to the patients themselves (Yokoo et al., 2009).

Canine iPS cells have been labelled with a fluorescent reporter gene and iron oxide and injected autologously into the left ventricular myocardial wall, allowing cells to be tracked using PET/CT and MRI scanning and allowing for further development of autologous transplantation of canine iPS cells. Furthermore canine iPS cells were differentiated toward endothelial cells and used in mouse models of hindlimb ischaemia and induced myocardial infarction. After 14 days there was a significant improvement in neo-vascularisation in the hindlimbs of treated mice, and an improvement of systolic function following delivery of iPS cells to border zones of infarcts at both 14 and 28 days. However, in both experiments the delivered iPS cells were found to have a significant death rate by week 6 post-implantation (Lee et al., 2011). This preliminary data implies that canine iPS cells may play a role in the repair of infarcted tissue, and has provided a tracking system has been devised to allow visualization of injected cells.

3.5 Conclusion

Cardiac disease is of huge importance in dogs, and plays a large part within small animal veterinary practice. Furthering the understanding of the physiology behind many of the diseases would enable the development of new treatment protocols. Stem cells may pave the way for these discoveries, and may in fact have a direct therapeutic role themselves. ES cells have several limiting issues, such as teratoma formation, and production of canine ES cells has proved difficult. Canine iPS cells have only recently been described, therefore the basic science of these cells must be identified prior to their use for cardiac research. This therefore leads to the further investigation of adult stem cells for the

investigation and treatment of cardiac disease. Preliminary findings using stem cells therapeutically have shown a short term benefit, but further work investigating the basic science of these cells, and the species specific differences needs to be performed before longer term therapeutic applications. Canine cardiac stem cells and mesenchymal stem cells will be discussed in more detail in Chapters 4 and 5 respectively. This is followed by Chapter 6 describing murine mesenchymal stem cells as a comparison to canine cells.

CHAPTER 4

Canine Cardiac Stem Cells

Abstract

Cardiac disease is a significant cause of morbidity and mortality in dogs and humans. In this chapter we are going to discuss cardiac specific ASCs including how they are defined and differentiated in the literature, and then describe our work isolating and characterising canine adult cardiac stem cells (CSCs) followed by our results and discussion. Specifically atrial cardiac explants were taken from dogs *post-mortem* and cultured to isolate CSCs. These cells were able to survive successive passages in serum free media, forming cardiospheres and were capable of clonal expansion under controlled culture conditions, demonstrating their ability for self-renewal. Characterisation of the CSCs demonstrated expression of c-Kit, GATA 4 and Flk-1 with no expression of cardiac lineage markers. Cardiac directed differentiation was performed based on the published literature and subsequent analysis demonstrated that partial cardiac directed differentiation was achieved with an increase in the expression of Nkx2.5 and cardiac troponin T. A second differentiation experiment was performed using indirect co-culture of CSCs with mesenchymal stem cells which appeared to drive the CSCs toward an endothelial lineage. The data in this study suggests that canine adult

CSCs are directly comparable to human stem cells and are multipotent, and therefore may be a source of cells for *in vitro* cardiac research.

4.1 Introduction

4.1.1 Isolation and Characterisation of Cardiac Stem Cells

The heart was long thought to be a terminally differentiated organ, with no capacity for repair. However, in the last 15 years this has been disproven, and the heart found not only capable of limited repair, but also to contain a resident stem cell population which may contribute to this repair (Beltrami et al., 2003, Messina et al., 2004).

4.1.1.1 Cardiospheres and Cardiac Stem Cells

Cardiospheres were first described by Messina *et al* in 2004. A phase-bright cell population grew out of cardiac explants in tissue culture, which could be harvested and grown in serum-free stem cell culture conditions. Under these conditions the cells formed spheres and were capable of clonal expansion. Furthermore it was found that the spheres contained a mixed population of cells, ranging from true stem cells through partially differentiated cells, fully differentiated cardiomyocytes and cells of vascular lineages (Messina et al., 2004). Murine cardiospheres were capable of spontaneous differentiation and this initial work was rapidly followed by more work utilising this stem cell isolation technique and the investigation of differentiation techniques for other species. It was shown that cardiosphere derived cells from the human and pig displayed properties suggestive of differentiation when co-cultured with rat neonatal cardiomyocytes (RNCM), including displaying calcium transients (Smith et al., 2007).

More recently an investigation into refining the technique to isolate CSCs from humans, pigs, mice and rats found that the original explant technique with only minor alterations consistently and reproducibly produced CSCs from all species investigated (Davis et al., 2009). CSCs from different species have been found capable of multipotent differentiation along three key routes; cardiac, vascular smooth muscle and endothelial lineages using several differentiation techniques (see Table 4.1 below) (Messina et al., 2004, Bearzi et al., 2007, Smith et al., 2007).

4.1.1.2 Origin of Cardiac Stem Cells

It was classically believed that the heart developed as a tube which folded to form the four main chambers of the heart. However, more recently it has been discovered that cardiac development is more complex, with variation seen in several key cardiac markers determining developmental fate. Wu *et al* proposed that a common precursor was present in the embryonic heart which later developed into the three main lineages based upon varied marker expression (Wu et al., 2006). Furthermore it was suggested that cells destined for the endothelial lineage broke away relatively early in development, whereas smooth muscle and cardiac cells remained closely related until later stages. Based upon genetic fate mapping it has now been shown that the heart develops in two stages, primary and secondary heart fields. During development the primary heart field forms the major area of the cardiac crescent, with secondary heart field cells present at extra-crescent edges. During development bi-lateral cardiac crescents fuse, at which point secondary heart field cells migrate into primary heart field

regions. The secondary heart field later divides into two regions, anterior and posterior. The anterior region becomes the right ventricle and outflow tract, whilst the posterior region becomes the atria and becomes continuous with the pro-epicardium. It has been found that CSCs are located throughout the heart, with primary heart field progenitors predominantly in the left ventricle and secondary heart field progenitors in the outflow tracts. This correlates with heterogeneity in marker expression based upon developmental origin (Figure 4.1) (Nakano et al., 2008, Domian et al., 2009).

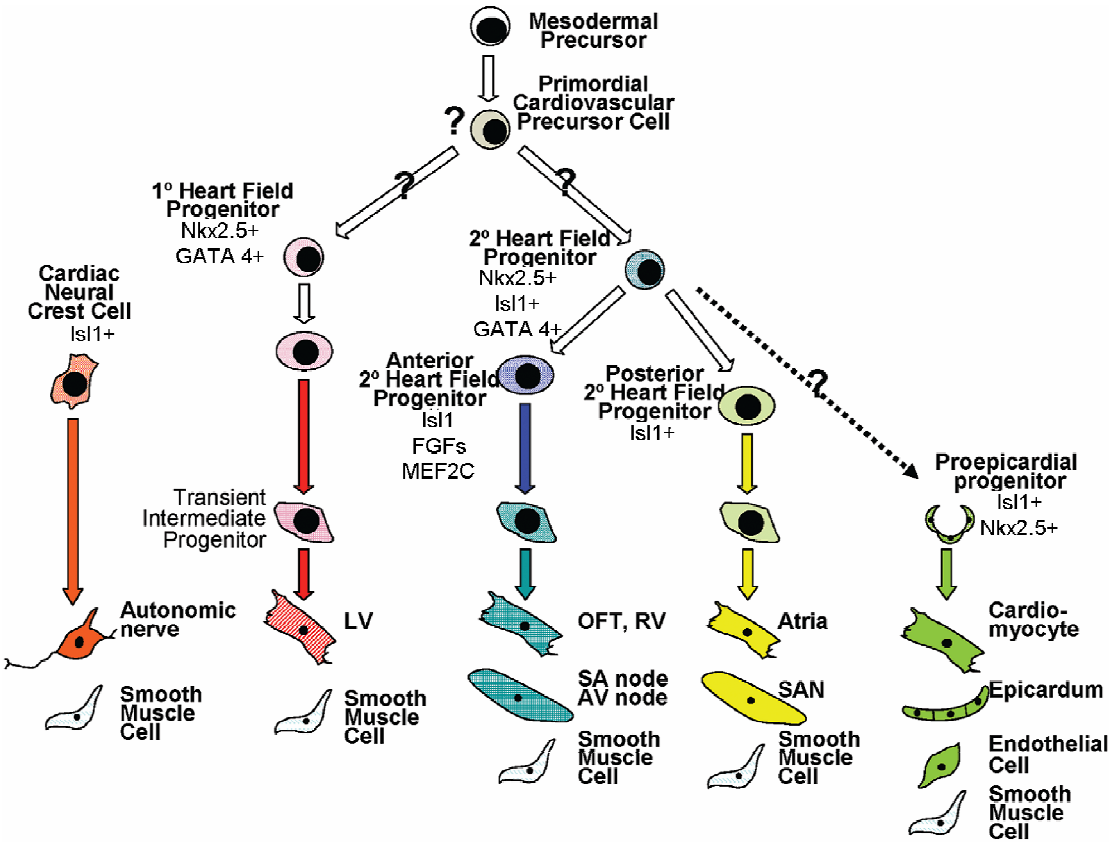


Figure 4.1. Developmental origin and marker expression of progenitor cells from different heart fields during cardiac growth. The heart develops from a single primordial cell from the mesoderm, and develops through two heart fields, primary and secondary. Marker expression alters during development through these fields with progenitor populations residing in tissues developed from both heart fields. Isl1, Islet 1; LV, left ventricle; OFT, outflow tract; RV, right ventricle, SA node and SAN, sinoatrial node; AV node, atrioventricular node. Taken from Nakano *et al* (Nakano et al., 2008).

4.1.1.3 Cardiac Stem Cell Niche

CSCs are found in discrete clusters within the interstitium of the heart, alongside differentiated somatic cells with cell to cell contact between the CSCs and the myocytes and fibroblasts. This niche is similar to that found in bone marrow; for example, in bone marrow the osteoblasts and stromal cells function as supporting cells, much like the myocytes and fibroblasts. Urbanek et al found that stem cell niches in the adult mouse heart were dispersed throughout the myocardium; however there was a twofold increase in volume of niche in the atria when compared to the ventricle. It was also found that the number of niches per mm^3 of atrial and apical myocardium was approximately 8-fold higher than at the base mid-region (Urbanek et al., 2006). Similarly the human right atria has significantly more c-Kit positive and islet 1 positive cells than the other three chambers within the heart. Furthermore, there is no overlap between cells expressing c-Kit and islet 1 suggesting that the two populations are separate, with more c-Kit positive cells present than islet 1 positive. Interestingly, there are more c-Kit positive cells within the hearts of female patients compared to male and there is an increase in c-Kit positive cell number with decreasing health of the heart, suggestive of an innate repair system having been triggered (Itzhaki-Alfia et al., 2009).

4.1.2 Defining Stem Cells

Stem cells have been defined as cells which are capable of self-renewal, and which have the ability to differentiate into multiple cell lineages. Stem cell markers have been used

to try to define stem cells *in vitro,* and also to help determine what stage of differentiation the cell may have reached. However, this is a developing and challenging area; defining the marker or groups of markers which can be reliably used to define a cell as a stem cell is a developing field. This is made more complicated by the possibility of interspecies differences and differences between different stem cell populations and stages of differentiation. A commonly used set of stem cell markers are Nanog, Oct 4 and STAT 3 – each playing a critical role in self-renewal and pluripotency.

4.1.2.1 Stem Cell Markers

4.1.2.1.1 Oct 4

Oct 4 is a POU transcription factor which is crucial for regulation of cell fate in the early embryo. It has a highly preserved octamer homeodomain with a sequence ATGCAAAT (Pesce and Scholer, 2001).

Oct 4 is expressed in the inner cell mass, and down-regulated post implantation in mice and human embryos (Cavaleri and Scholer, 2003). However, Kirchhof et al found that in the bovine and porcine species, Oct 4 was not only expressed in the inner cell mass (the totipotent cells) but also in the trophoectoderm; therefore Oct 4 expression differs between species and is not only restricted to pluripotent cell expression (Kirchhof et al., 2000).

Oct 4 is hypothesised to have a gene dosage effect, controlling cell renewal at lower levels. Over-expression has been found to trigger ES cell specification toward cardiac and mesodermal development whereas Oct 4 repressed cells develop into trophoectoderm. Oct 4 knockout mouse embryos are able to develop to the blastocyst stage, however the inner cell mass cells are not pluripotent (Nichols et al., 1998, Niwa et al., 2000, Chambers et al., 2003, Hou et al., 2007, Stefanovic and Puceat, 2007, Wang et al., 2008).

Oct 4 can be also be found expressed within adult stem cells, and therefore has a general use as a marker of stem cells (Beltrami et al., 2007). However, Oct 4 expression has also been demonstrated in some adult human differentiated cells such as human peripheral blood mononuclear cells (Zangrossi et al., 2007).

Cellular distribution of Oct 4 varies between cell lines. In general within stem cell cultures, Oct 4 has a nuclear location, corresponding to its function as a transcription factor critical for self-renewal. However, Oct 4 has also been demonstrated within the cytoplasm; Zangrossi *et al* found Oct 4 expression in the cytoplasm of peripheral blood mononuclear cells; Cheng *et al*, found that cancerous germ cells expressed Oct 4 in the cytoplasm; whereas primordial germ cells displayed a typical nuclear expression leading them to the hypothesis that expression pattern may reflect the transition to a tumour cell line (Cheng et al., 2007, Zangrossi et al., 2007)..

4.1.2.1.2 STAT 3

Signal transducer and activator (STAT) proteins play a key role in cytokine signal transmission, mediated by the interleukin family of proteins. STAT 3 is activated by the interleukin-6 family of cytokines, including leukaemia inhibitory factor (LIF) and functions through receptor complexes containing the signal transducer gp130. These in turn activate the JAK pathways, thereby transmitting a cell membrane signal to the nucleus. Recruitment and translocation of STAT 3 to the nucleus modifies gene expression to sustain self-renewal (Niwa et al., 1998, Matsuda et al., 1999, Burdon et al., 2002).

STAT 3 is not ES cell specific but can be found in a number of other cell types. It can be associated with cell movement such as leukocyte, epidermal and keratinocyte migration. It is also associated with cellular differentiation, such as B cell differentiation into antibody forming plasma cells and may play a role in neoplasia, where persistent activation triggers tumourigenesis (Bromberg and Darnell, 2000, Hirano et al., 2000).

4.1.2.1.3 Nanog

First described in 2003, Nanog (named after ‘Tir nan Og’, the Celtic land of the ever-young) works in parallel with STAT 3 to drive ES cell self-renewal, and blocks the differentiation effects of Oct 4. Also, by suppression of differentiation, Oct 4 expression is maintained (Chambers et al., 2003). Over-expression of Nanog promotes pluripotency

and this function is controlled by Nanog-Nanog dimer formation (Wang et al., 2008). Nanog has been found to be down-regulated transiently in mouse embryos upon differentiation, and is thought to predispose cells to differentiate, but does not mark commitment. Interestingly, it is possible to culture pluripotent cells in the self-renewing state without Nanog, however it is essential for the formation of germ cells (Chambers et al., 2007). Adult stem cells have been described as positive for Nanog, suggesting the importance of this marker for pluripotency in certain cell populations (Beltrami et al., 2007).

4.1.2.2 Cardiac Stem Cell Markers

More specific markers for CSCs have been described. These markers have been used in isolation and in panels to isolate specific progenitor populations from the heart itself.

4.1.2.2.1 Islet 1

Islet 1 is a developmental lineage marker which labels a cell population that contributes to the embryonic heart secondary heart field (Figure 4.1), including most cells of the right ventricle, both atria, specific regions of the left ventricle and the outflow tract. In mice lacking islet 1 the outflow tract, right ventricle and a large proportion of the atria are missing, indicating the importance of this marker in cardiac development (Cai et al., 2003). Furthermore genetic variation in islet 1 has been associated with non-syndromic congenital heart disease (Stevens et al., 2010). Laugwitz *et al* identified cells expressing

islet 1 in rat, mouse and human myocardium, and found mouse islet 1 positive cells capable of differentiation into the cardiomyocyte phenotype; mES cells isolated based on islet 1 expression were found to co-express Nkx2.5, and were capable of differentiating into cardiac, smooth muscle and endothelial cell lineages (Laugwitz et al., 2005, Moretti et al., 2006). Human islet 1 positive ES cells are also capable of differentiating along all three major lineages during embryoid body development (Bu et al., 2009).

4.1.2.2.2 Stem cell antigen 1 (Sca 1)

Sca 1 has been used to isolate a cell population from the adult heart which can differentiate *in vitro* into cardiomyocytes; Oh *et al* found that Sca 1 positive cells isolated from the mouse were negative for cardiac structural genes and c-Kit and Nkx2.5, but positive for GATA 4 (Oh et al., 2003); Moretti *et al* found that cells isolated from the mouse had relatively high expression of CD45 (~40%) and low expression of c-Kit (~10%) (Moretti et al., 2006). Sca 1 is a commonly used marker for isolation of haematopoietic stem cells in the mouse. It is a member of the murine Ly6 gene family and encodes an 18 kDa glycosyl phosphatidylinositol-anchored cell surface protein. It has been proposed that Sca 1 may act as a co-signalling molecule, which modifies the signalling capacity of a receptor complex, making the signal more efficient. A number of potential human homologs for murine Ly6 have been identified, however a direct homolog for Sca 1 has not been found (Holmes and Stanford, 2007). However, murine Sca 1 antibody has successfully been used to isolate a cell population from human

cardiac biopsies which are capable of high levels of differentiation (80-90%) into spontaneously beating cardiomyocytes when exposed to the de-methylating agent 5'Azacytidine (5'AZA) and transforming growth factor β1 (TGFβ1) *in vitro* (Goumans et al., 2007, Smits et al., 2009b).

4.1.2.2.3 C-Kit

C-Kit was first used to isolate a population of cells from the adult heart with properties of CSCs (Beltrami et al., 2003). C-Kit (CD117) is a stem cell marker and cells taken from the heart expressing this marker are capable of differentiating into cardiomyocytes (Beltrami et al., 2003, Kubo et al., 2008, Tallini et al., 2009, D'Amario et al., 2011). C-Kit has been used in a number of different species to define a CSC, including the mouse (Beltrami et al., 2003, Tallini et al., 2009) and the human (Bearzi et al., 2007, Aghila Rani et al., 2008). Wu *et al* found that isolating mES cells co-expressing c-Kit and the cardiac lineage marker Nkx2.5 identified a cell line capable of long-term *in vitro* expansion that was capable of differentiating into cardiomyocytes and smooth muscle precursors (Wu et al., 2006). Kubo found that there was an increased abundance of c-Kit positive cells in failing hearts of humans compared to normal hearts, and there was frequent co-expression of the haematopoietic marker CD45 (Kubo et al., 2008).

4.1.2.2.4 Flk-1

Flk-1 is also known as KDR (kinase insert domain receptor) and VEGFR-2 (vascular endothelial growth factor receptor 2). Flk-1 in combination with c-Kit has been used to isolate a population of CSCs which differentiated preferentially into endothelial and smooth muscle cells (D'Amario et al., 2011). It has also been shown that Flk-1 positive, c-Kit negative ES cells have the capacity for differentiation into all three major cardiac lineages (Yang et al., 2008) and Flk-1 positive, CXCR4 positive ES cells are enriched for cardiac progenitor cells (Nelson et al., 2008). It has also been proposed that Flk-1 in combination with islet 1 may define a population of cells with a preference for differentiation into endothelial and smooth muscle (Moretti et al., 2006). It has been suggested that Flk-1 positive cells are developmental precursors of the endocardium in the developing heart and can also be precursors of skeletal myocytes (Motoike et al., 2003, Misfeldt et al., 2009).

4.1.2.2.5 Nkx2.5

Nkx2.5 is a homeobox gene expressed early as part of the cardiac lineage (Martin et al., 2004). Homeobox genes are part of a superfamily encoding transcription regulatory proteins which act at critical points in development. The distinguishing feature of this family of genes is a structurally conserved DNA-binding motif known as the homeodomain, which contains the genetic data for proteins which are sequence specific DNA-binding factors. Murine Nkx2.5 is a protein of 318 amino acids, and Lints et al

found it to be heavily distributed in foetal and adult heart tissues, and also in NIH3T3 fibroblasts. When Nkx2.5 expression was compared to that of α-cardiac actin and β-myosin heavy chain (β-MHC), it was found to be expressed prior to both *in vivo*, and *in vitro*, and could therefore be used as an early marker of cardiac myogenesis (Lints et al., 1993).

4.1.2.2.6 GATA 4

GATA 4 is part of the GATA family of transcription factors, known to have a regulatory effect upon differentiation, growth and survival of a diverse range of cell types. The family contains 6 proteins, GATA 1-6. GATA 1, 2 and 3 are important regulators of haematopoietic cells and their derivatives. GATA 4, 5 and 6 have a role in mesodermal and endodermal derived tissues. They are a highly conserved family of proteins throughout evolution, with members present in fungi, worms, flies, frogs, fish, chickens, mice and humans (Cantor and Orkin, 2005) and share a related zinc finger DNA-binding domain (Svensson et al., 1999).

GATA 4 is one of the earliest transcription factors expressed in developing murine heart cells, and is expressed abundantly in cardiac myocytes during the animal's lifespan (Heikinheimo et al., 1994). It has been shown to be a major binding factor in the developing heart, with over-expression increasing differentiation of beating cardiomyocytes and inhibition preventing differentiation and triggering apoptosis. In GATA 4 knockout mice embryonic development fails, thought to be due to an

endodermal defect. In GATA 4 deficient mice (GATA 4 null chimera with C57/Black 6 mice) the cardiomyocytes are able to differentiate, and late cell markers are expressed including atrial natriuretic peptide (ANP) and α-myosin heavy chain (α-MHC) (Kuo et al., 1997). GATA 6 deficient ES cells show abnormal differentiation, and an increase in GATA 4 levels, so it is suggested that GATA 6 may regulate GATA 4 expression and GATA 4 is necessary for endodermal differentiation, but may not be essential for cardiomyocyte differentiation, where GATA 6 may replace it (Morrisey et al., 1998). GATA 4 and 6 share high homology in amino acid sequence, approximately 80-90% which may account for this effect (Morrisey et al., 1997). Murine GATA 6 gene contains an Nkx2.5 binding element essential for cardiac-specific expression of the promoter during cardiogenesis (Molkentin et al., 2000). GATA 4 has been shown to co-operate with a number of other transcription factors *in vitro* including MEF2, Nkx2.5 and GATA 6 (Pikkarainen et al., 2004).

GATA 5 is detected during embryonic development of the heart, lungs, bladder, urogenital ridge and the gut, and in adult gut, bladder, lungs, endocardium, but not the myocardium (Morrisey et al., 1997).

4.1.3 Canine Adult Cardiac Stem Cells

Canine adult stem cells have been isolated from the myocardium using fluorescence activated cell sorting (FACS) with antibodies for c-Kit, Sca 1 and MDR 1 (an ABC-transporter found in side-population cells). These cells were negative for lineage

markers for haematopoietic, cardiac and skeletal muscle and were capable of self-renewal. Following culture in differentiation media clonal cells differentiated into cells phenotypically resembling cardiomyocytes, endothelial cells, smooth muscle cells and fibroblast lineages. The cardiomyocyte-like cells expressed GATA 4, GATA 6, MEF2C and Ets 1 – all factors of cardiac cell lineages (Linke et al., 2005). Cardiospheres were generated using canine CSCs, plated onto a growth substrate poly-l-ornithine. These cells were isolated from ventricular tissue and cultured in a stem cell media, containing growth factors, but no serum. The spheres grew up to ~230 μm diameter after 8 days in culture. Early spheres, around day 2-3, were strongly immunoreactive for stem cell markers c-Kit and pax 6; later spheres, around day 6-8 strongly expressed cardiomyocyte specific markers – cardiac myosin heavy chain, cardiac troponin I (CTI) and cardiac sarcomeric actinin, as well as those for vascular endothelial cells – von Willebrand factor (vWF), and vascular smooth muscle cells-smooth muscle actin (SMA). Co-culture of the spheres with RNCMs promoted differentiation into both cardiomyocytes and also vascular cell phenotypes (Bartosh et al., 2008).

4.1.4 Cardiac Stem Cells in Disease Treatment

Adult stem cells derived from human endomyocardial biopsies cultured to form cardiospheres have been used to examine the therapeutic use of stem cells *in vivo*. The cardiosphere derived cells were injected into the border zone of acute myocardial infarcts in immunodeficient mice and the injected cells migrated into the infarct zone,

and were still present after 20 days. The percentage of viable myocardium within the infarct zone was higher in the cardiosphere derived cell treated group and this group also had a higher left ventricular ejection fraction when compared to the non-treated animals (Smith et al., 2007). Oh *et al* showed that Sca 1 positive adult CSCs from the mouse homed to the myocardium when injected intravenously using a cre/lox system bound to the α-MHC gene. This showed that the cells relocated to the myocardium, and differentiated to express α-MHC – however it was impossible to discern if this expression was caused solely by differentiation of the injected cells, or by some level of cell fusion (Oh et al., 2003). Laflamme *et al* injected human cardiomyocytes derived from ES cells into infarcted rat hearts and found an improvement in contractile function and a limitation in dilation (Laflamme et al., 2007). Smits *et al* injected human CSCs, and cardiomyocytes derived from CSCs into the border zone of infarcts in mice and found that both cell populations improved cardiac function equally over three months, indicating that pure stem cell populations may be used therapeutically, avoiding the lengthy *in vitro* differentiation procedures which may increase cellular mutation. Crucially it was seen that these cells did not fuse with the murine cells, thereby suggesting that it was a differentiation effect rather than cell fusion causing the improvements seen (Smits et al., 2009a).

4.1.4.1 Native Cardiac Stem Cell Activation

Realistically the therapeutic use of CSCs is always going to be limited due to the difficulty in isolation from cardiac tissue and the limited expansion potential *in vitro*.

However, activation of the endogenous stem cell population *in situ* would bypass the need to remove the CSCs. Linke *et al* demonstrated in a canine model the activation of resident cardiac stem cells in chronically diseased hearts using the growth factors Insulin like Growth Factor (IGF-1) and Hepatocyte growth factor (HGF) (Linke et al., 2005). It has also been demonstrated that priming of the Wilms tumour gene (Wt1) by peptide thymosin β4 (Tβ4) appears to drive a mild repair response in damaged myocardium most likely from cells of epicardial origin (Smart et al., 2011). Adopting this strategy would circumvent the need to remove stem cells prior to re-implantation, overcoming a number of the issues outlined above and is an area for future development.

4.1.5 Differentiation of Cardiac Stem Cells

CSC differentiation *in vitro* has been described in various species using a variety of culture techniques and pharmacological interventions and importantly a variety of defining criteria varying from the development of spontaneous beating to the expression of one or more structural proteins (Table 4.1).

Table 4.1. Differentiation of cardiac stem cells using different species and techniques. CNX 43–connexin 43; CTI–cardiac troponin I; CTT-cardiac troponin T; β-MHC–β myosin heavy chain; α-MHC–α myosin heavy chain; MLC-2a-myosin light chain 2a; MLC-2v-myosin light chain 2v; vWF–von Willebrands Factor; SMA–smooth muscle actin., α-SA–α-sarcomeric actinin

Author	*Year*	*Cell source*	*Technique*	*Markers before*	*Markers after*	*Beating*
***Beltrami* et al**	2003	Human	Differentiation media; MEM + serum + dexamethasone	GATA 4, Nkx2.5, MEF2C	GATA 4, Nkx2.5, MEF2C	No
***Messina* et al**	2004	Human	Direct co-culture with RNCM	Flk-1, c-Kit, CD34	CTI, CNX 43	No
***Laugwitz* et al**	2005	Mouse	Direct co-culture with RNCM	Islet 1	α-SA, CTT	No
***Goumans* et al *and* *Smits* et al**	2007 / 2009	Human	Differentiation media; IMDM:DMEM Hams F12 + ITS+ premix + NEA + serum + 5'AZA + ascorbic acid + TGFβ1	Sca 1, c-Kit, islet 1, GATA 4, Nkx2.5, MEF2C	CTT, β-MHC, cardiac actin, α-SA, CTI,	Yes
***Oh* et al**	2003	Mouse	Differentiation media; M-199 + serum + 5'AZA	Sca-1	α-SA, CTI, Nkx2.5, α-MHC, β-MHC	No
***Gaetani* et al**	2009	Human	Exposed to low frequency electro-magnetic fields calcium ion cyclotron energy resonance	CTI, Nkx2.5, MHC, CNX 43, SMA	Increased: CTI, Nkx2.5, MHC, CNX 43	No
***Matsuura* et al**	2004	Mouse	Differentitaion media; IMDM + serum + 5'AZA or Oxytocin	Sca 1, Nkx2.5, GATA 4	Nkx2.5, GATA 4, MEF2C, β-MHC, MLC-2a, MLC-2v Cardiac α-actin	Yes
Bartosh *et al*	2008	Canine	Direct co-culture with RNCM	C-Kit, MHC, α-SA, CTI, vWF SMA		Yes

(Beltrami et al., 2003, Oh et al., 2003, Matsuura et al., 2004, Messina et al., 2004, Laugwitz et al., 2005, Goumans et al., 2007, Bartosh et al., 2008, Gaetani et al., 2009, Smits et al., 2009b).

4.1.5.1 Cardiomyocyte Differentiation Mechanisms and Pathways

The Wnt pathway is believed to be significant in the developing heart (Figure 4.2). It has been suggested that Wnt has a variable effect dependent upon both stage of differentiation, subset of progenitor cell, and dosage (Nakano et al., 2008). Notch is required to negatively regulate β-catenin of the Wnt pathway and allow cardiac progenitor cells to move from expansion toward differentiation (Kwon et al., 2009). DKK1, a Wnt inhibitor and Wnt11 and Wnt5A of the Wnt pathway were upregulated during cardiac differentiation on human ES cells using END-2 co-culture suggesting the importance of these factors during differentiation (Beqqali et al., 2006). Islet 1 precursors are predominantly found in the secondary heart field and β-catenin is a key upstream regulator required to drive islet 1 expression in cardiac precursors (Cai et al., 2003, Lin et al., 2007). Islet 1 triggers differentiation into cardiac cells by limiting self-renewal and interacts with STAT 3, and may control its activity in gene expression modulation (Hao et al., 2005, Kwon et al., 2009).

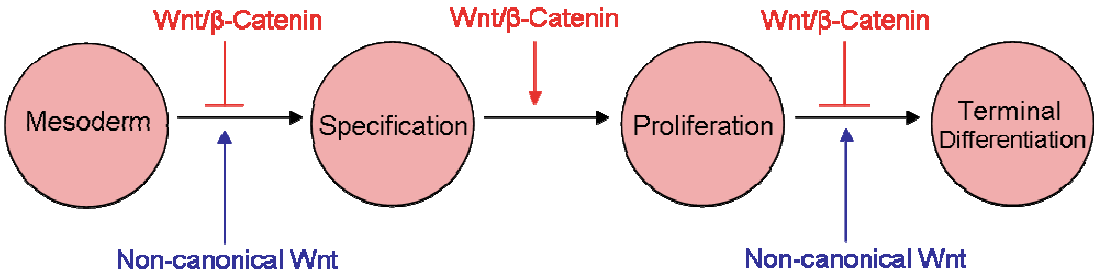


Figure 4.2. Cardiac differentiation controlled by the Wnt pathway. Canonical Wnt involving β-catenin, and non-canonical Wnt closely regulate developmental stages of cardiac differentiation via controlling terminal differentiation where activation and inhibition determine proliferation or terminal differentiation (Gessert and Kuhl, 2010).

4.1.6 Clinical Trials

Very few clinical trials exist using CSCs as the therapeutic agent. This may be due to the difficulty in isolating the cells and difficulty in *in vitro* expansion thereby limiting available cell numbers for treatment. To date four trials are underway (Table 4.2).

Table 4.2. Clinical trials currently using cardiac stem cells (www. Clinicaltrials. Gov).

Trial Name	*Trial*	*Location*	*Start and End*
SCIPIO	Cardiac Stem Cell Infusion in Patients with Ischaemic Cardiomyopathy	University of Louisville, Kentucky, USA	February 2009-December 2010
CADUCEUS	Cardiosphere-Derived Autologous Stem Cells to Reverse Ventricular Dysfunction	Ceders-Sinai Medical Centre, Johns Hopkins University, USA	May 2009-May 2011
ALCADIA	Autologous Human Cardiac-Derived Stem Cells to Treat Ischaemic Cardiomyopathy	Kyoto Prefectural University of Medicine, Japan	April 2010-March 2013
TICAP	Transcoronary Infusion of Cardiac Progenitor Cells in Patients with Single Ventricle Physiology	University of Okayama, National Cardiovascular Centre, Japan	January 2011-January 2013

Results of these trials are awaited with great anticipation. Stem cell therapy in heart disease is hoped to be a successful future treatment, and has already been performed with alternative cell sources such as mesenchymal stem cells (discussed in Chapter 5; Mesenchymal Stem Cells). There is optimism regarding the use of ASCs for therapeutics with short term improvements seen as previously discussed. However it will be interesting to compare the results using CSCs to those obtained with MSCs to see if there is superior therapeutic potential given the difficulties in isolating CSCs.

4.1.7 Aims

This chapter investigates the isolation and culture of canine primary CSCs grown from harvested cardiac tissue. The CSCs will be characterised in terms of morphology, culture

behaviour and marker expression. Furthermore the CSCs will be differentiated toward cardiac lineages using two separate published techniques and a modified protocol of each to act as a control, and the resultant cell populations analysed for evidence of differentiation.

4.2 Materials and Methods

4.2.1 Cardiac Stem Cell Isolation

4.2.1.1 Tissue Samples

Full thickness canine cardiac tissue was harvested from the right and left atria (including auricles) and ventricles immediately post mortem from dogs euthanized for clinical reasons, and with owners' consent. Tissue was placed immediately into chilled cardiac explant media (CEM); Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen, UK. Catalogue number 21056) supplemented with 10% fetal calf serum (Invitrogen, UK), 100 U/mL Penicillin G and 100 μg/mL Streptomycin (both Invitrogen, UK); 2 mmol/L L-glutamine (Gibco, UK); 0.1 mmol/L 2-Mercaptoethanol (2-ME; Sigma, UK) and transported directly to the laboratory for processing. In addition, sections of myocardium were taken and stored immediately in RNAlater (Qiagen, UK) at -20 °C to use for whole heart RNA extraction.

4.2.1.2 Tissue Preparation

Cardiac tissue was dissected into 1-2 mm^3 pieces, washed with Ca^{++} and Mg^{++} free PBS and digested with 0.2% trypsin (Invitrogen, UK) and 0.1% collagenase IV (Invitrogen, UK) for 5 minutes at 37 °C; this was repeated 3 times. The tissue explants

were rinsed with CEM media. The tissue was then placed into Fibronectin (Costar, Cambridge) coated T25 flasks with CEM media and cultured at 37 °C in 5% CO_2.

4.2.1.3 Isolation of Cardiac Stem Cells

Explants generally became adherent to the flasks over a period of 3-4 weeks and generated an adherent fibroblast layer. In addition, small phase bright non-adherent cells appeared over the fibroblasts. These cells either appeared singly or arranged themselves into free-floating grape-like clusters or were loosely attached to the fibroblast layer. The phase bright cells were harvested by pooling the CEM media containing the phase bright cells with two PBS washes. The cells obtained usually ranged in number from 2×10^4 to 3×10^5 and were considered to be CSCs.

4.2.2 Cardiac Stem Cell Culture

Harvested CSCs from explant culture were counted and seeded onto 24 well plates at a total cell count of approximately 5×10^4 in 600 μL basic cardiosphere growing serum-free media which comprised: 35% IMDM; 65% Dulbecco's Modified Eagle's Medium (DMEM) – Ham F-12 mix (Invitrogen, UK. Catalogue number; 21331) supplemented with 2% 50X B27 (Invitrogen, UK); 0.1 mmol/L 2-ME; 10 ng/mL epidermal growth factor (EGF, Peprotech, UK); 20 ng/mL basic fibroblast growth factor (bFGF, Peprotech, UK); 40 nmol/L Cardiotrophin-1 (Invitrogen, UK); 40 nmol/L bovine thrombin (Sigma, UK); 100 U/mL Penicillin G; 100 μg/mL Streptomycin; 2 mmol/L

L-Glutamine. The cells were fed every 2-3 days by the addition of 2.5 ng/mL cardiotrophin; 1.5 ng/mL EGF and 1.5 ng/mL bFGF. Under these conditions cells either remained singly or formed large spherical clusters which are referred to as cardiospheres from approximately 7 days. CSCs were taken for analysis at days 0, 5, 10 and 15 from serum free culture from multiple dogs. Repeated harvests of viable CSCs were collected from the original T25 flasks, approximately every 1-2 weeks over a period of up to 4 months.

4.2.3 Cardiac Stem Cell Clonal Expansion

CSCs were harvested from explant flasks as described in 'Isolation of Putative Cardiac Progenitor Cells' (Chapter 2; Materials and Methods) and re-suspended in 1 mL of cardiosphere growing media. Cells were counted using a haemocytometer and trypan blue, and adjusted to a total cell count of 5 cells/mL. 96 well plates (TPP Tissue Culture Plates, USA) were coated with 0.1% gelatin and 100 μL of cell suspension was added to each well. Plates were incubated at 37 °C in 5% CO_2. Cells were passaged every 3-4 days by removing the media, and washing the attached cells once with PBS, and adding 100 μL of fresh cardiosphere media. Cell cultures were examined twice weekly, and wells with clonal expansion were monitored. At 80-90% confluence, media was removed from the cells and the cells washed once with PBS. Cells were removed from the gelatin with 0.05% Trypsin/EDTA (Invitrogen, UK), and re-suspended in cardiosphere growth media. Cells were initially spilt 1:2, and increased to a 1:5 split as cells numbers increased.

4.2.4 Magnetic Activated Cell Sorting (MACS®)

CSCs grown in either 24 well plate culture or T25 flasks in cardiosphere media were sorted using c-Kit (CD117) MACS® Microbeads (MiltenyBiotec, Germany). Separation buffer was prepared; PBS, pH 7.2 plus 0.5% FBS (Invitrogen, UK) and 2 mmol/L EDTA and filter sterilised. CSCs were harvested and counted and subsequently re-suspended in 300 μL of separation buffer. 50 μL of FcR blocking reagent and 50 μL c-Kit beads (both Miltenyi Biotec, Germany) were added to the cell suspension. The suspension was incubated for 30 minutes at 4 °C with rotation. The cells were then washed in 5 mL of cold separation buffer and centrifuged at 300g for 10 minutes, after which the supernatant was discarded. Cells were re-suspended in 1 mL of separation buffer and applied to a LS MACS column attached to a MACS Separator (both Miltenyi Biotec, Germany). The c-Kit negative fraction was removed from the column and placed into cardiosphere growing media. Following this the c-Kit positive fraction was obtained and placed into cardiosphere growing media. Both positive and negative fractions were then placed at 37°C in 5% CO_2 for continuous culture, or harvested for either RNA extraction or immunofluorescence.

4.2.5 D17 Canine Osteosarcoma Cell Culture as a Control Line

D17 (canine osteosarcoma) adherent cells and spheres were used as a positive control cell line. Adherent monolayer cells were cultured in RPMI 1640 (Invitrogen, UK) with

10% FBS (Invitrogen, UK) on standard tissue culture plastic. D17 spheres were cultured using ultra-low attachment six well plates (Corning, UK) containing 2 mL N2 medium with 1% methylcellulose. The N2 media consisted of 2 x Dulbecco's modified Eagle medium (DMEM)/F12 with progesterone (20 nmol/L), putrescine (100 mol/L), sodium selenite (30 nmol/L), transferrin (25 g/mL), insulin (20 g/mL) (Sigma Biochemicals) and two growth factors, human EGF (10 ng/mL) and human bFGF (10 ng/mL) (Peprotech). These cells were cultured as described previously by Wilson *et al* (Wilson et al., 2008).

4.2.6 Transcriptional Analysis of Cardiac Stem Cells

RNA was extracted and cleaned up from cell pellets of cultured CSCs from multiple dogs over 15 days. 50 ng of total RNA was reverse transcribed and PCR studies performed on two dogs as described in Chapter 2; Materials and Methods. Cells were analysed for the expression of; STAT 3, Nanog, c-Kit, islet 1 Flk-1, Nkx2.5, GATA 4 and cardiac troponin T. GAPDH was multiplexed in each reaction. Cardiac markers were optimized using whole heart tissue RNA, STAT 3 using D17 RNA and Nanog using canine ovarian tissue RNA. Primers were designed and prepared as described in Chapter 2; Materials and Methods (Table 4.3).

Table 4.3. Oligonucleotides for canine sequences. CTT-Cardiac Troponin T; Beta 1 Ad-β_1-adrenergic Receptor; Cardiac RyR-Cardiac Ryanodine Receptor; CTI-Cardiac Troponin I; VEGF-Vascular Endothelial Growth Factor; vWF-Von Willebrands Factor; SMA-Smooth Muscle Actin. F–Forward primer; R –Reverse primer.

Marker	Sequence 5'-3'	Size (BP)	Tm (°C)
STAT 3	F: GTG GAG AAG GAC ATC AGC AAT AA	250	56
	R: AAC TTG GTC TTC AGG TAT GGG GC		
Nanog	F: CAG AAG ATG AGG ACG GTG TTC TC	189	58
	R: CCA GTT GCT TTT CTG CCA CCT C		
C-Kit	F: ATA TCC CAA ACC GGA GCA C	193	58
	R: TCA CCG AAG AAT TGA CAT CG		
Islet 1	F: GGT TTC TCC GGA TTT GGA AT	183	57
	R: CAC GAA GTC GTT CTT GCT GA		
Nkx2.5	F: CCA AGG ACC CTC GAG CTG A	185	62
	R: CGA CAG ATA CCG CTG CTG CT		
GATA 4	F: CAA GAT GAA TGG CAT CAA CC	216	57
	R: GGT TTG AAT CCC CTC TTT CC		
Flk-1	F: CCA CCC AGA CTC AGC ATA CA	188	58
	R: CAC TTT TGG AAT CGT GAG CA		
CTT	F: GAA GGA CCT GAA CGA ACT	210	60
	R: CCT CCT GTT CTC CTC CTC CT		
Beta 1 Ad	F: GAG TAC GGC TCC TTC CTG TG	274	61
	R: AGC ACT TGG GGT CGT TGT AG		

Marker	Sequence 5'-3'	Size (BP)	Tm (°C)
Cardiac RyR	F: AAG CGA AGC AGC CCA AGG GT	888	62
	R: TCA GCA AAG TGT GCC GCG CT		
CTI	F: TCT GCG CAT GGC GGA TGA GA	624	62
	R: TTG CGG CCC TCC ATT CCA CT		
VEGF	F: TTC CTG CAG CAT AGC AAA TG	293	57
		(239,311)	
	R: AGG GAG GCT CCT TCT TCC AG		
vWF	F: CTG GGA GAA GAG AGT CAC GG	235	61
	R: GTG GAT GGA GTA CAC GGC TT		
SMA	F: GGG GAT GGG ACA AAA GGA CA	525	61
	R: GCC ACG TAG CAG AGC TTC TCC TTG A		
Glomulin	F: TTG AAG AGC CCT CTG GAA AA	131	57
	R: ACT CCA AAG GGT GGA CAA TG		
Connexin 43	F: *ATG AGC AGT CTG CCT TTC GT*	249	58
	R: TCT GCT TCA AGT GCA TGT CC		
GAPDH	F: CAT CAA CGG GAA GTC CAT CT	428	Various
	R: GTG GAA GCA GGG ATG ATG TT		

4.2.7 Translational Analysis of Cardiac Stem Cells

4.2.7.1 Immunofluorescence

CSCs were cultured on 4 well chambered slides as described in Chapter 2; Materials and Methods. Cells were fixed and primary antibodies to c-Kit (Dako, UK. Cat no. A4502; previously validated in the canine (Morini et al., 2004)) and Flk-1 (Abcam, UK. Cat no. Ab2349; cross-reactive with canine) were diluted at a 1:100 in PBST (0.1% normal goat serum; 0.1% Tween 20 in PBS) and placed on the cells overnight in a humidified chamber at 4 °C. The following day the cells were washed, and labelled with secondary antibody Alexafluor 488 conjugated goat polyclonal anti-rabbit IgG (Invitrogen, UK), at 1:500, for 1 hour at room temperature. Nuclei were subsequently labeled using a DAPI mount (Vectastain, Vector Labs, UK) and slides stored at -20 °C.

4.2.7.2 Western Blot

CSCs were isolated and harvested as previously described. Following 12 days in serum free stem cell culture the cells were harvested and protein extracted as previously described. Protein was quantified using Bradford analysis, and loaded into an SDS page gel and transferred to a nitrocellulose membrane.

The blot was then probed using three rabbit polyclonal primary antibodies; anti-Oct 4, anti-STAT 3 and anti-Nanog (Abcam, UK. Catalogue numbers: ab19857, ab30646,

ab21603 respectively) diluted at 1:500, 1:250 and 1:250 respectively and left overnight at 4 °C. Mouse monoclonal primary antibody to beta-actin (Abcam, UK. Catalogue number: ab6276) was used as a loading control, diluted at 1:2000. Swine polyclonal to rabbit IgG horseradish peroxidase conjugated secondary (Dako, UK. Catalogue number PO217) and Goat whole molecule IgG antibody to mouse secondary antibodies (Sigma-Aldrich, UK. Catalogue number A-3562) were used at 1:1000 dilution and left at room temperature for 1 hour. Biorad ECL chemiluminescent detection system (Biorad, UK) was used to detect protein bands, and results visualised by exposure to radiographic film.

4.2.8 Cardiac Directed Differentiation of Cardiac Stem Cells

CSCs were harvested from explant flasks as previously described. A 48 well plate was coated with 0.1% gelatin, and 2 x 10^4 cells were placed into each well. Two dogs were used for the studies, and two published differentiation protocols were followed for each dog – that described by Smits *et al* (Smits et al., 2009b) and that described by Oh *et al* (Oh et al., 2003). Triplicate wells for each dog following each protocol were plated, plus triplicates of a modified standard protocol as a differentiation control, and a triplicate of cells maintained in cardiosphere media for each dog was run as a negative control. Initially all wells were placed into cardiosphere media for 5 days, with half media changes every 2 days, to establish stem cell colonies. At that point the individual protocols were initiated.

4.2.8.1 Differentiation based upon Smits *et al*

Following 5 days in stem cell culture, cardiosphere media was removed and cells gently washed twice with PBS. Fresh cardiosphere media was added to control stem cell wells. Basic differentiation media was added to all differentiating cell wells; 235 mL of IMDM and 235 mL of Ham's F12 nutrient mixture with GlutaMAX-I , 10 mL FBS, 1 x (5 mL) MEM nonessential amino acids, 1 x (5 mL) insulin-transferrin-selenium (all Invitrogen, UK), and the cells were cultured in this media for 24 hours. At this point the media was removed, and fresh differentiation media was added including 5 μmol/L 5' AZA (Sigma, UK). For a further 2 days 5 μmol/L 5'AZA was added, at which point media was removed and the cells were washed in PBS, followed by 2 days of standard differentiation media. Following this, media was refreshed every 2 days, with 17.5 μg/mL of ascorbic acid (Sigma, UK) added. For standard differentiation 1 ng/mL of TGFβ1 (Peprotech, USA. Catalogue number 100-21C) was added every 4 days; a modified protocol without adding TGFβ1 was performed alongside. This was continued for 17 days, (Figure 4.3) at which point cells were harvested and pelleted, with the triplicates pooled to increase cell numbers, generating an average. Throughout the experiment CSC control wells were cultured alongside, and had cardiosphere media refreshed at each media change.

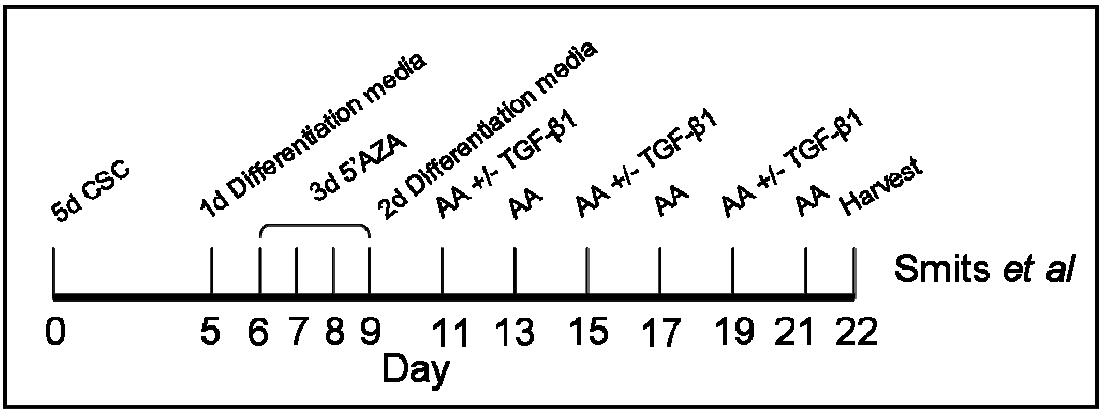


Figure 4.3. Differentiation protocol based upon Smits *et al*. Cardiac stem cells (CSCs) were differentiated using 5'AZA and ascorbic acid (AA) with and without transforming growth factor-β (TGFβ1) over 22 days.

4.2.8.2 Differentiation based upon Oh *et al*

Following 5 days in stem cell culture, cardiosphere media was removed and cells gently washed twice with PBS. Fresh cardiosphere media was added to control wells and M-199 media (Cambridge Bioscience, UK) with 10% FBS was added to the differentiating wells and all wells left for 3 days with daily media changes. After 3 days the media was removed and replaced with M-199 media containing 2 % FBS with 3 μmol/L 5'AZA. Fresh 2% serum M-199 media was added after 3 days of 5'AZA treatment and media refreshed every 3 days for a total of 17 days (Figure 4.4), at which point cells were harvested and pelleted as above. A modified protocol was run alongside

which did not have 5'AZA added to the M-199 media. Furthermore control cardiac stem cell wells had cardiosphere media refreshed at each media change.

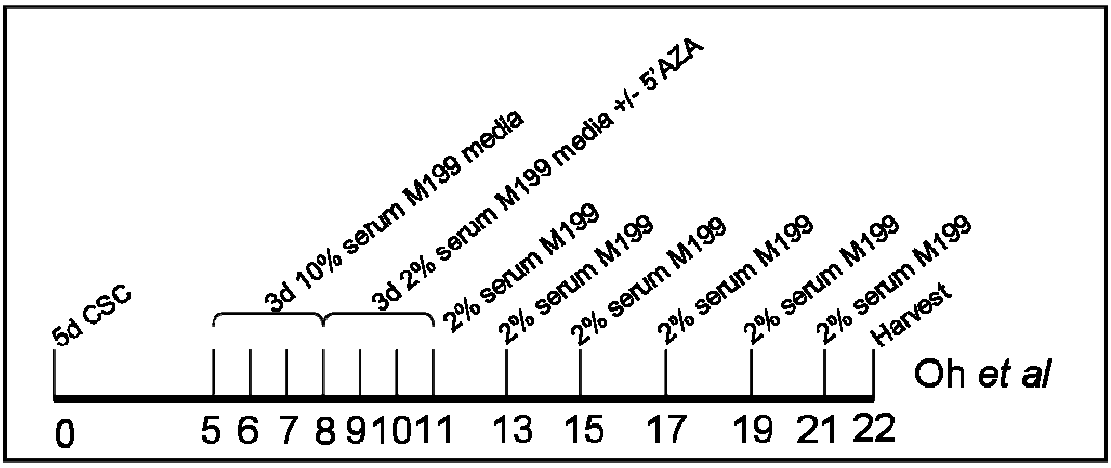


Figure 4.4**.** Differentiation protocol based upon Oh *et al.* CSCs were differentiated in M-199 media with and without 5'AZA over 22 days.

Pooled cell pellets were snap frozen and stored at - 80°C. RNA was extracted and reverse transcribed as described in Chapter 2; Materials and Methods and used downstream for semi-quantitative and quantitative PCR using primers described in Table 4.3.

4.2.8.3 Quantitative RT-PCR Analysis of Cardiac Directed Differentiation

Primers and probes for GATA 4, Flk-1 and CTT were designed and prepared as described in Chapter 2; Materials and Methods and primers ordered from MWG

Eurofins and probes from Roche. Nkx2.5 primers and probe were designed using the Applied Biosystems software, and ordered pre-bound to probe at a 20 x concentration from Applied Biosystems. Primer and probes sequences are described in Table 4.4.

Table 4.4. Primer sequences and probe product information for quantitative RT-PCR for CTT, GATA 4, Flk-1 and Nkx2.5.

Target	*Forward*	*Tm (°C)*	*Reverse*	*Tm (°C)*	*Size (BP)*	*Probe Number (Roche, UK) or sequence*
CTT	AGG AGG AGG AGC TGA TTT CAC	59	CTC CTT CTC CCG CTC GTT	60	95	132
GATA 4	ACG GAA GCC CAA GAA CCT TA	60	CAG GAG GAA GGC TCT CAC C	59	71	62
Flk-1	CAC CAC ATC CGC TGG TAC T	59	GGT TTG TCA TTA AGA CAG CTT GG	59	73	25
Nkx2.5	CAA GGA CCC TCG AGC TGA T	53	AGC TCC ACC GCC TTC TG	53	57	AAG AAA GAG CTG TGC TCA CTG

4.2.8.3.1 cDNA Synthesis

Extracted RNA from cardiac differentiation pellets was reverse transcribed as described in Chapter 2; Materials and Methods.

4.2.8.3.2 PCR Master Mix

A master mix was prepared for CTT, Flk-1, GATA 4, Nkx2.5 and 18s rRNA controls as described in Tables 4.5, 4.6 and 4.7.

Table 4.5. Target gene master mix for CTT, Flk-1 and GATA 4

Master mix	*1 x (µL) (n x =number of wells)*
Realtime Master mix	5
10 x Primer/Probe (FAM)	0.3

Table 4.6. Target gene master mix for Nkx2.5

Master mix	*1 x (µL) (n x =number of wells)*
Realtime Master mix	5
20 x Primer/Probe (FAM)	0.15
Water	0.15

Table 4.7. Target gene master mix for 18s rRNA control gene

Master mix	*1 x (µL) (n x =number of wells)*
Realtime Master mix	5
20 x 18s VIC Control	0.15
Water	0.15

4.2.8.3.3 Primer Efficiency and Optimisation

Real time plates and cover foils were ordered from Roche, UK. RNA was extracted from canine whole heart tissue for use as a positive control and 100 ng of total RNA was used for reverse transcription to cDNA. Neat cDNA was serially diluted 1:2.5, 1:5, 1:10 and 1:50 and a plate plan was devised for primer pair optimisation (Figure 4.5).

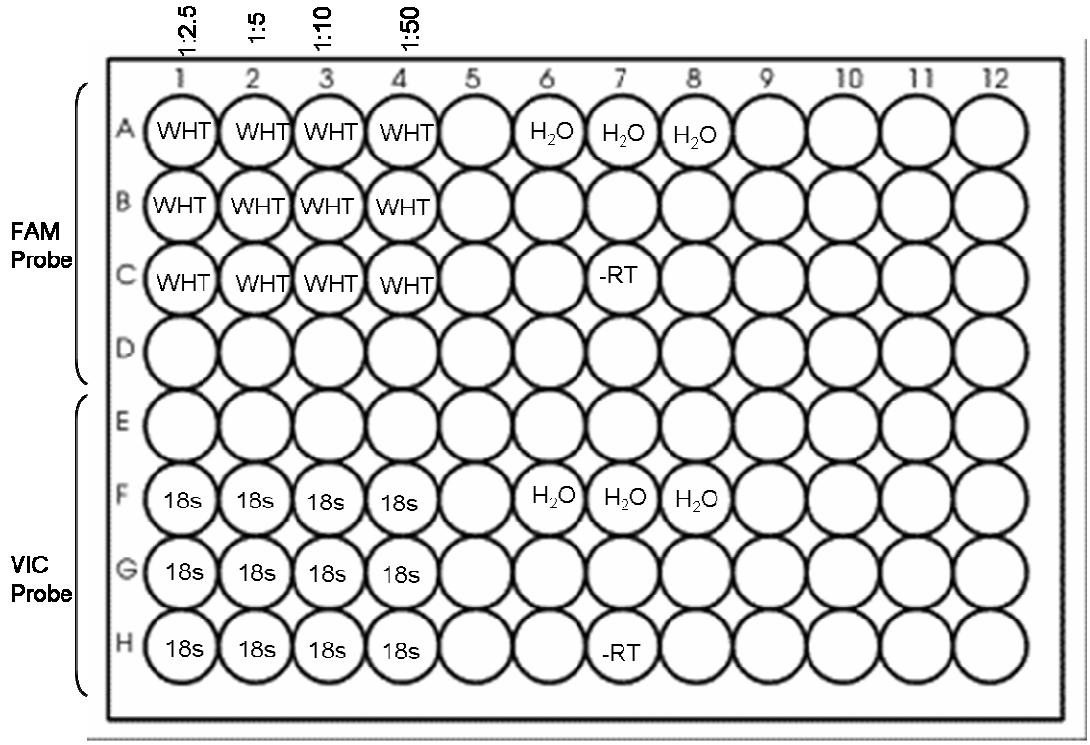


Figure 4.5. 96-well plate plan for CTT, GATA 4, Flk-1 and Nkx2.5 primer optimization. Wells were designed to allow for triplicates of whole heart tissue (WHT) cDNA dilutions (1:2.5, 1:5, 1:10, 1:50). Triplicate wells of water negative controls were run, and –RT wells of sample RNA were run with each primer. Primers for CTT, GATA 4, Flk-1 and Nkx2.5 were bound to a FAM probe, and primers for 18s rRNA in-house gene positive control bound to a VIC probe.

5.3 μL of appropriate master mix (FAM; CTT, Flk-1, GATA 4 or Nkx2.5. VIC; 18s rRNA) and 4.7 μL of appropriate cDNA dilution was added to each well. 4.7 μL of diluted sample RNA (-RT) or 4.7 μL of DEPC treated water was added to each control well. Wells were sealed shut using a foil and centrifuged at 1500g for 2 minutes to pool

samples into base of wells. Plates were placed into the Roche Lightcycler 480 and run on a relative analysis programme as described in Table 4.8:

Table 4.8. Programme data for Roche Lightcycler 480

Programme	*Cycle*	*Acquisition*
Pre-incubation	1	N
Amplification	50	Quantify
Cooling	1	N

With individual cycles as described in Table 4.9:

Table 4.9. Temperature settings per cycle for Roche Lightcycler 480

Programme	*Temperature (°C)*	*Acquisition*	*Hold*	*Ramp*
Pre-Incubation	95	N	10 minutes	4.4
Amplification (50 x)	95	N	10 seconds	4.4
	54	N	30 seconds	2.2
	72	Single	1 second	4.4
Cooling	40	N	10 seconds	1.5

Data was exported to Microsoft® Excel and a primer efficiency curve was generated including an R^2 value, which gave an indication of reaction efficiency (a value closer to 1 is more efficient).

4.2.8.3.4 Relative Gene Expression Analysis

Following primer optimization samples could be compared for relative expression of specific gene targets. 100 ng of total RNA of each sample from dog A and B Oh protocols were reverse transcribed and the subsequent cDNA analysed at a 1:5 dilution. A plate plan for relative expression is shown in Figure 4.6.

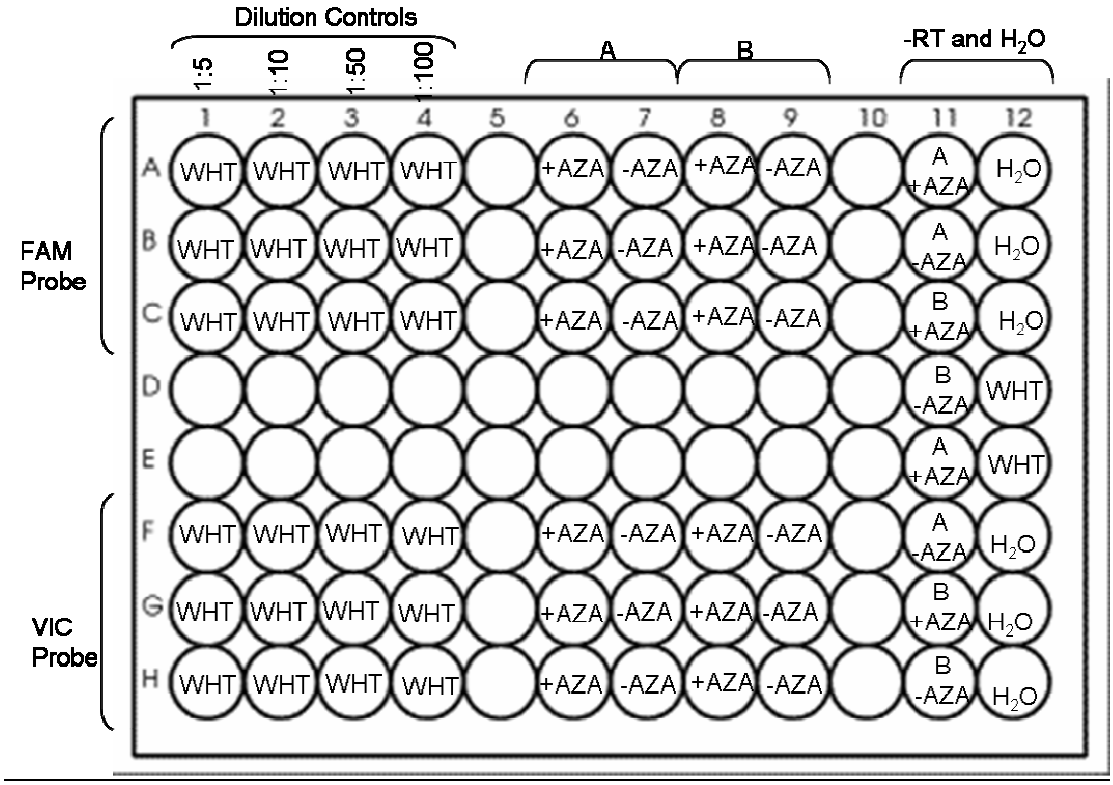


Figure 4.6. 96-well plate plan for gene expression analysis. Wells were designed to allow for triplicates of sample cDNA from both dogs A and B with (+ AZA) and without (-AZA) 5'AZA. Triplicate wells of WHT cDNA were run alongside in multiple dilutions. Diluted RNA and water controls were run concurrently. Primers for CTT, Flk-1, GATA 4 and Nkx2.5 were bound to a FAM probe, and primers for 18s rRNA in house gene positive control bound to a VIC probe.

The plate design allowed for comparison between differentiated (+AZA) and undifferentiated (-AZA) samples in two dogs, A and B following the Oh protocol. Whole heart tissue cDNA was added to allow for a reference control sample. All

negatives were run on the same plate using the same master mix that was used for the samples.

4.2.9 Cardiac Stem Cell Co-Culture with Mesenchymal Stem Cells

Canine CSCs were grown in indirect co-culture with autologous MSCs using a transwell system.

4.2.9.1 Harvest of Cardiac Stem Cells and Mesenchymal Stem Cells

CSCs and MSCs were harvested as described in Chapter 2; Materials and Methods and placed in DEM media and counted.

Following the plate plan in Chapter 2; Materials and Methods, 2 x 10^4 CSCs were seeded into base wells of the 24 well plate (growth area 1.9cm^2) in 600 μL of DEM media, with 1 x 10^4 MSCs seeded onto transwell inserts (growth area 0.33 cm^2) in 100 μL DEM media. Control wells of CSCs alone were cultured alongside. Plates were then placed at 37 °C in 5% CO_2. Day 0 CSCs were pelleted at this point, and snap frozen and stored at -80 °C. Images and harvests from both co-cultured and control wells were taken at days 6 and 10 post-plating and cells pelleted and snap frozen and stored at -80 °C. RNA was subsequently extracted from pellets and used for semi-quantitative RT-PCR analysis as described in Chapter 2; Materials and Methods for all markers in Table 4.3.

4.3 Results

4.3.1 *In Vitro* Cardiac Explant Culture

Cardiac tissue explants were taken *post-mortem* and cultured to enable outgrowth of putative stem cells. Explants become anchored to the base of the flasks within 3-4 weeks and an adherent fibroblast layer grew out from the explant. Varying numbers of spherical phase bright cells were also apparent in the culture media and appeared either singly or in grape like clusters; either free floating or loosely adherent to the underlying fibroblast layer (Figure 4.7). We defined these cells as CSCs based upon their characterization and published literature (Messina et al., 2004). Subjectively, cell numbers peaked between 2nd and 3rd harvest and atrial explants consistently produced more phase bright cells compared to the ventricles, and were therefore used for all the results in this study.

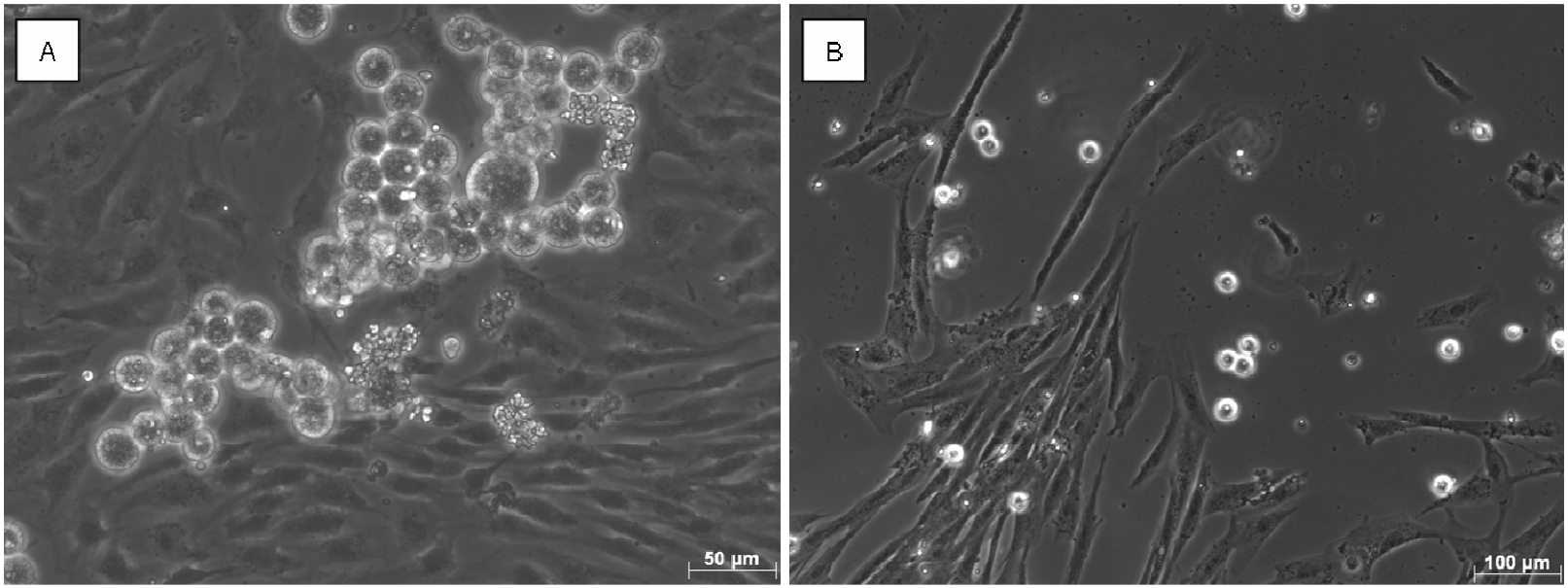


Figure 4.7. Cardiac explant culture. Large round phase bright cells migrated from cardiac explants over a layer of fibroblast like cells, and were often found in clusters free floating in the media or lightly attached to the fibroblast layer (magnification A, x 200; B, x 100).

4.3.2 *In Vitro* Culture of Cardiac Stem Cells

Following harvest from explant culture CSCs were placed into serum free stem cell media. Over a 2-3 week culture period cells maintained their large circular size, typically forming cardiospheres (Figure 4.8). Over time these cardiospheres continued to increase in size and number, and could be harvested, and passaged. CSCs could be maintained in serum free culture conditions for up to 4 weeks, with partial media changes every 2-3 days.

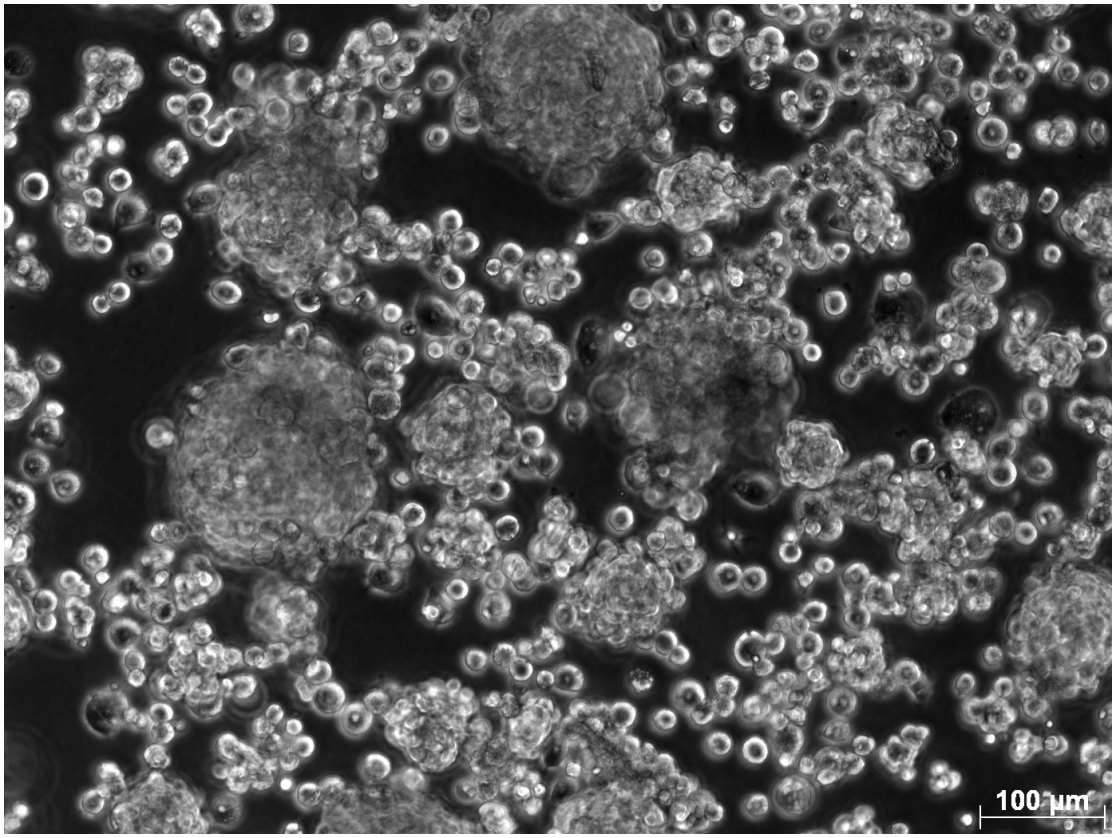


Figure 4.8. Cardiospheres in culture. CSCs formed large clusters in serum free culture conditions appearing morphologically like cardiospheres (magnification x 100).

4.3.3 Clonal Expansion of Cardiac Stem Cells

To demonstrate clonal expansion, a characteristic of stem cell populations (Messina et al., 2004), the CSCs were plated at low density. Single cells adhered to gelatin within 12 hours following low density cell plating. Over 5-7 days these individual cells produced large spherical colonies indicative of clonal expansion. These spheres could be passaged, creating more spheres. At passage, a large central cell could be seen, surrounded by cells of varying sizes, suggestive of asymmetric division (Figure 4.9).

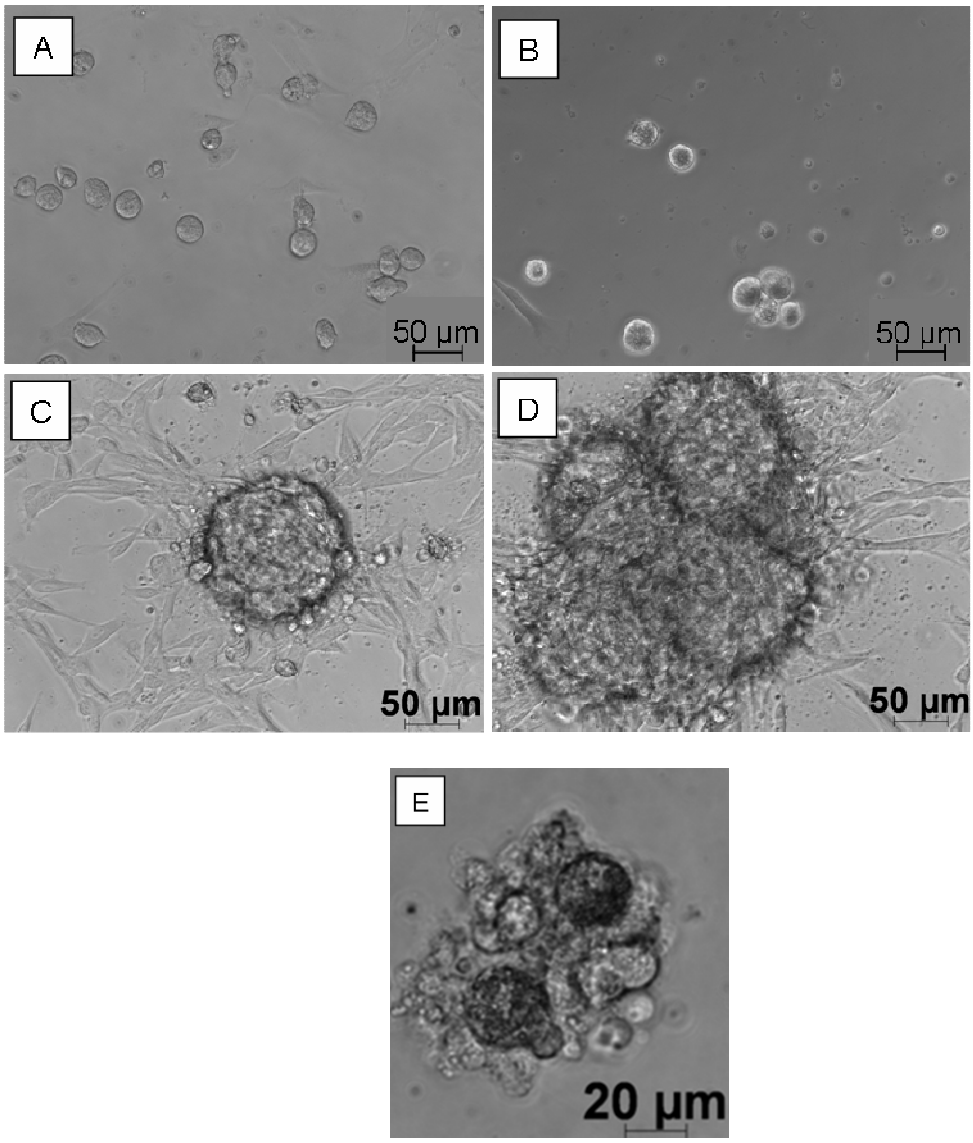


Figure 4.9. Clonal expansion of CSCs. The cells were capable of clonal expansion in serum free culture conditions; CSC plated at low cell number could be seen as individual stem cells in adherent culture (A and B) which formed spheres over 7 days (C and D) (A-D, magnification x 200). Passage of cardiospheres following clonal expansion (E) demonstrated cells of varying sizes surrounding a large central cell resembling a CSC (magnification x 400).

4.3.4 Characterisation of Cardiac Stem Cells

4.3.4.1 Oct 4, Nanog and STAT 3 Stem Cell Markers

4.3.4.1.1 Western Blot

CSCs were analysed for their expression of the pluripotency stem cells markers Oct 4, Nanog and STAT 3 using western blotting of protein extracted from cardiosphere cultures and both adherent and sphere forming D17 cells as a control. Following extraction, total protein was quantified using Bradford analysis. Low levels of total protein were extracted (0.09 μg/mL) from cardiosphere pellets, and therefore a limited amount of protein was loaded into the blot (approx 0.6 μg total). Blots were probed for approximately 45 hours using primary antibody for Oct 4, Nanog and STAT 3, with secondary antibody exposure lasting 90 minutes at room temperature. All three antibodies identified protein bands in the two canine protein samples and both the adherent and sphere D17s. STAT 3 appeared at the predicted size of 88 kDa in all cell lines (Figure 4.10). However, both Nanog and Oct 4 appeared at a larger size than that predicted (100 kDa versus 39 kDa and 75 kDa versus 43 kDa respectively) and was therefore considered to be non-specific binding (data not shown). Beta-actin controls indicated equal loading of all cell line protein samples.

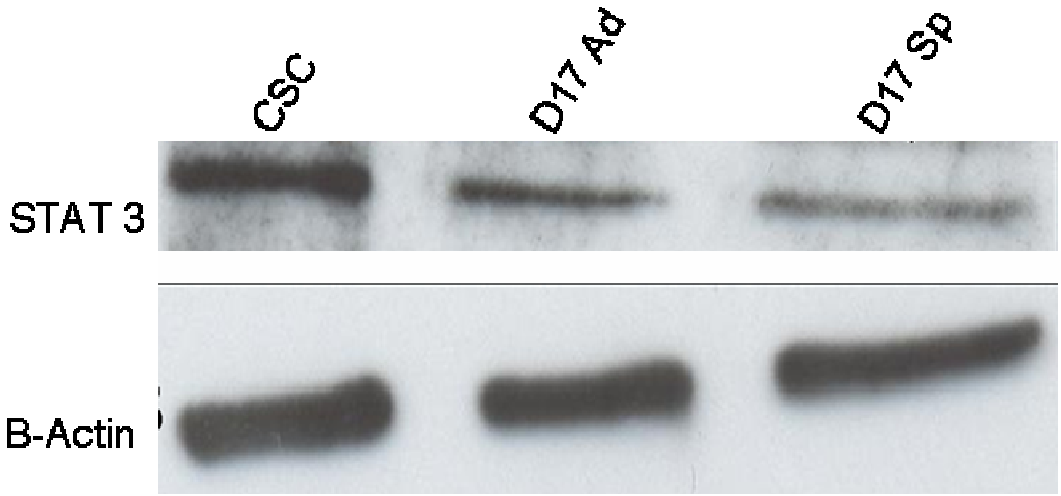


Figure 4.10. Western blot analysis of STAT 3 expression in cardiospheres (CSC) and D17 cell lines; adherent (D17Ad) and sphere (D17Sp). STAT 3 at expected product size 88kDa. Beta-actin loading control at expected size 42kDa.

4.3.4.1.2 Immunofluorescence for Oct 4, Nanog and STAT 3

Cardiosphere cultures were examined for Oct 4, Nanog and STAT 3 expression using immunofluorescence. A strong fluorescent signal for Oct 4 could be detected in both cardiosphere cells and adherent D17s (control cells). Staining appeared to be both nuclear and cytoplasmic and the majority of cells were Oct 4 positive in both CSCs and control cells (Figure 4.11. Images donated by Dr Sally-Anne Argyle). Nanog fluorescence was also present in CSCs, however in contrast to Oct 4, the staining was weaker and only small numbers of cells in each field were stained positive (Figure 4.12).

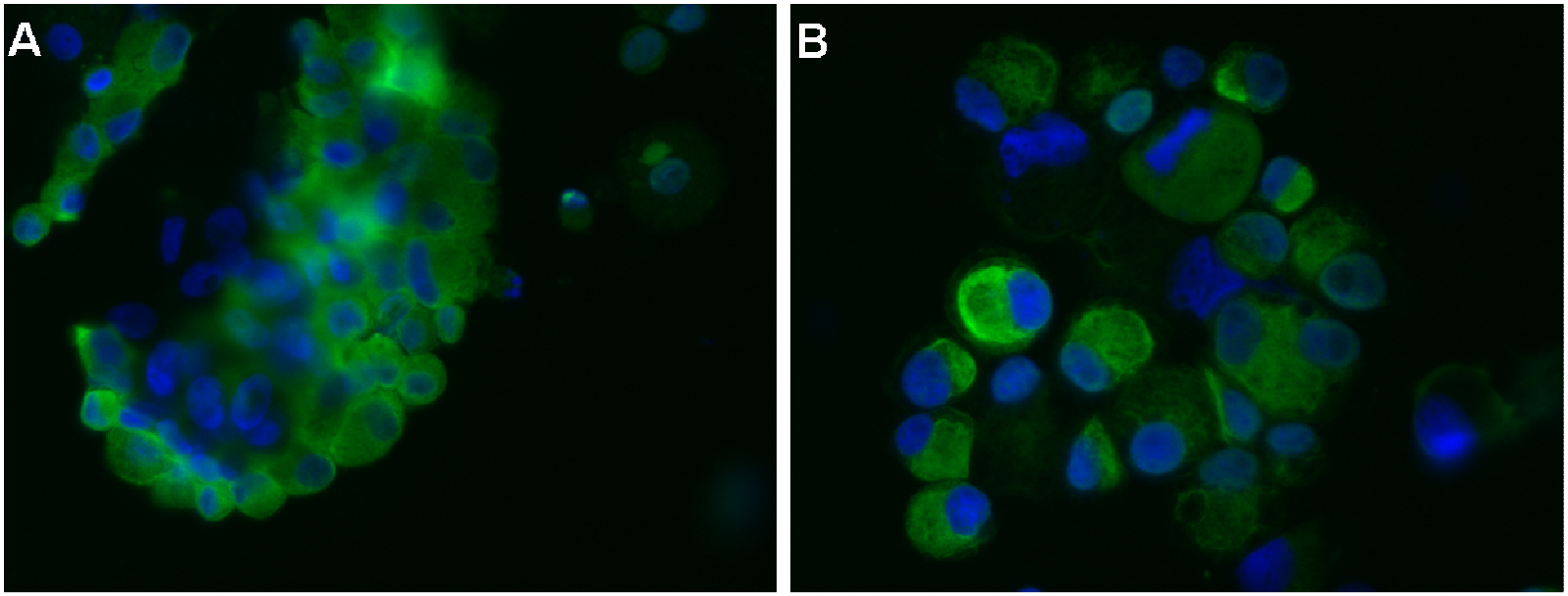


Figure 4.11. Immunofluorescence analysis of CSC cultures for Oct 4 following growth in cardiosphere media with adherent D17 cells for positive control. Oct 4 (FITC) is seen distributed heavily within both (A) Cardiospheres (B) D17 cells. Oct 4 labelled with FITC, nuclei counterstained with DAPI. Magnification x 400.

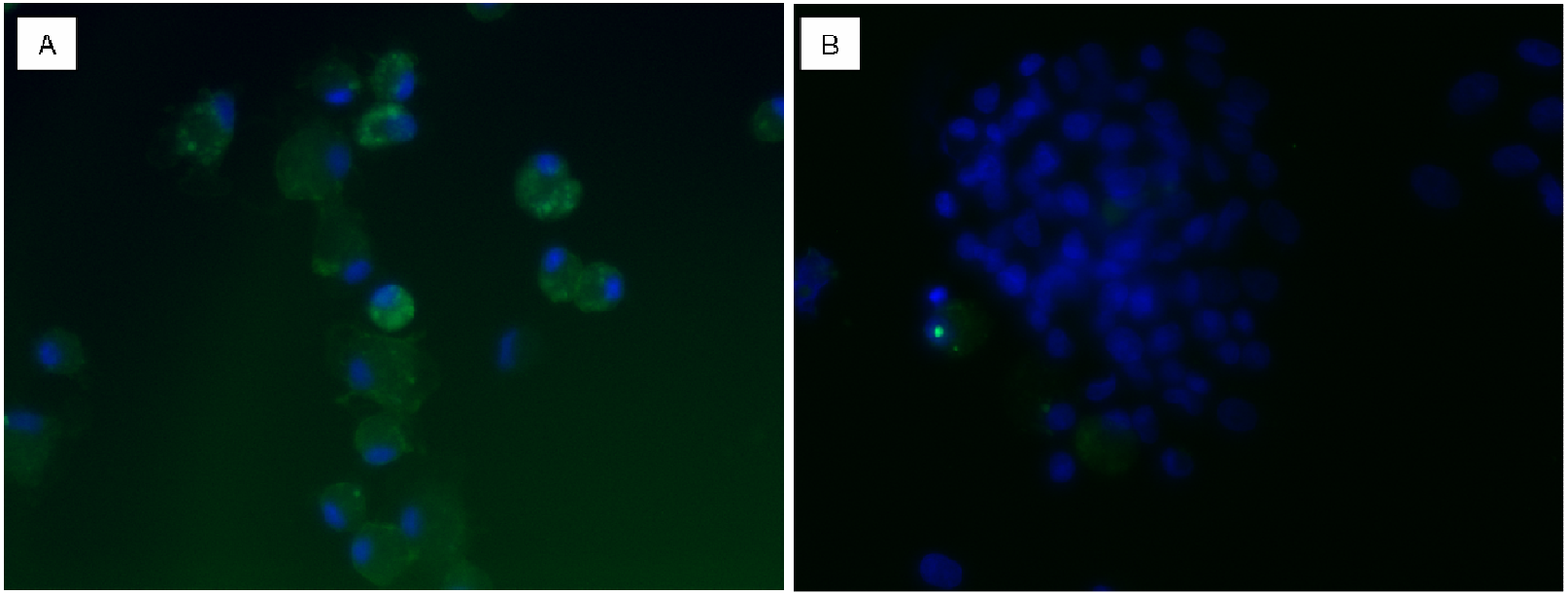


Figure 4.12. Immunofluorescence (IFA) analysis of CSC cultures following growth in cardiosphere media. CSC cultures were positive for Nanog within cytoplasm (FITC, A), nuclei counterstained for DAPI. Negative control of secondary antibody alone were run alongside (B) showing positive nuclei labelling with DAPI. Magnification x 200.

4.3.4.2 Cardiac Stem Cell Markers

4.3.4.2.1 Expression at Transcriptional Level

Cellular harvests from multiple dog explant cultures were placed into cardiosphere culture media and subsequently harvested at different time points, over a 15 day period. Cellular marker expression levels were examined compared to whole heart tissue using an internal GAPDH positive control as a multiplex reaction. Over the time course there was very little change in expression patterns across all markers, indicating that the cells were maintaining their expression profiles over time within the cardiosphere media when examined with two dogs (Figure 4.13). Furthermore, comparing expression of the same markers using multiple dogs demonstrated little variation between CSCs harvested from different dogs (Figure 4.14). Due to experimental limitation, some dog CSCs were analysed at multiple time points, whereas others were analysed at only one harvest. STAT 3 levels remained high throughout the time course, and had similar levels to the whole heart. C-Kit levels appeared high throughout the time course, and higher for the stem cells than for whole heart tissue with some variation between dogs and time points. Islet 1 was not expressed in CSC populations with the exception of dog 10 at day 6, and dog 8 at day 15 and in these isolated cases the cultures appeared to have a reduced expression of c-Kit and Flk-1. We were unable to analyse these populations further. Flk-1 appeared to have high expression across all time points, with a suggestion of lesser expression in whole heart. Both Nkx2.5 and CTT were only expressed in the whole heart cultures, whereas GATA 4 was expressed at relatively constant levels across all the time

points, and with a similar level in the whole heart. It was seen that for the time 0 analysis there appeared to be a reduced level of expression for all markers, including the standardised control GAPDH; GAPDH levels returned to a constant high level from day 5 analysis onwards. All samples were negative for the stem cell marker Nanog (data not shown).

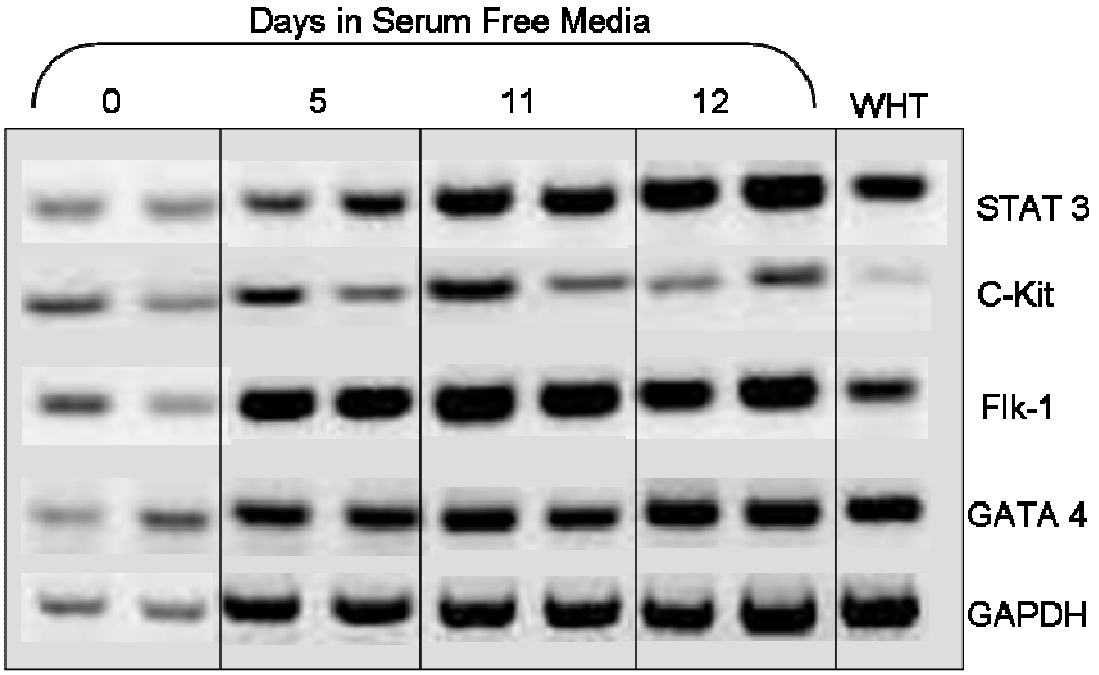


Figure 4.13. Semi-quantitative RT-PCR of extended culture CSCs from two dogs. Cells were cultured in serum free media and examined for marker expression at a transcriptional level. No difference was seen with either dog in marker expression over 15 days when compared to whole heart tissue (WHT).

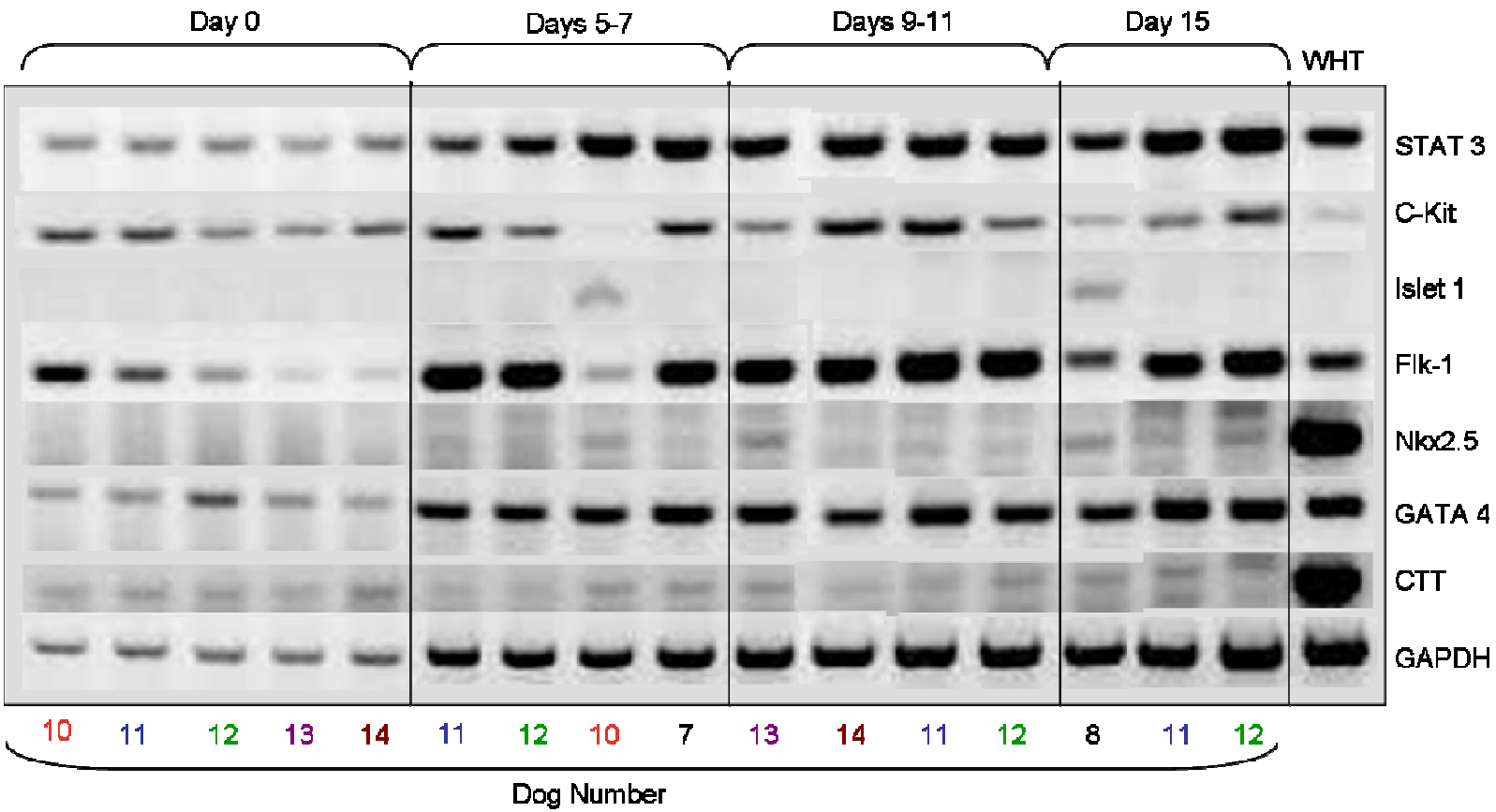


Figure 4.14. Semi-quantitative RT-PCR of extended culture CSCs from multiple dogs. Little difference in seen in marker expression when comparing between CSCs from different dogs over all time points examined (days in serum free media). Dog numbers are coloured, and some dogs have analysis for multiple time points, whereas some have only one time point analysis.

4.3.4.2.2 Immunofluorescence Imaging for C-Kit and Flk-1 of Cardiac Stem Cells

CSCs were grown on chambered slides on gelatin to allow cells to adhere. 24 hours following plating individual cells were fixed and labelled for c-Kit and Flk-1 expression using immunofluorescence (IFA) (Figure 4.15 and 4.16). C-Kit clearly had a cell surface and cytoplasmic distribution (Figure 4.15), whereas Flk-1 had a nuclear location, both as expected (Figure 4.16 A). DAPI was used to counterstain nuclei, and negative controls excluding primary antibodies were stained in parallel (Figure 4.16 B).

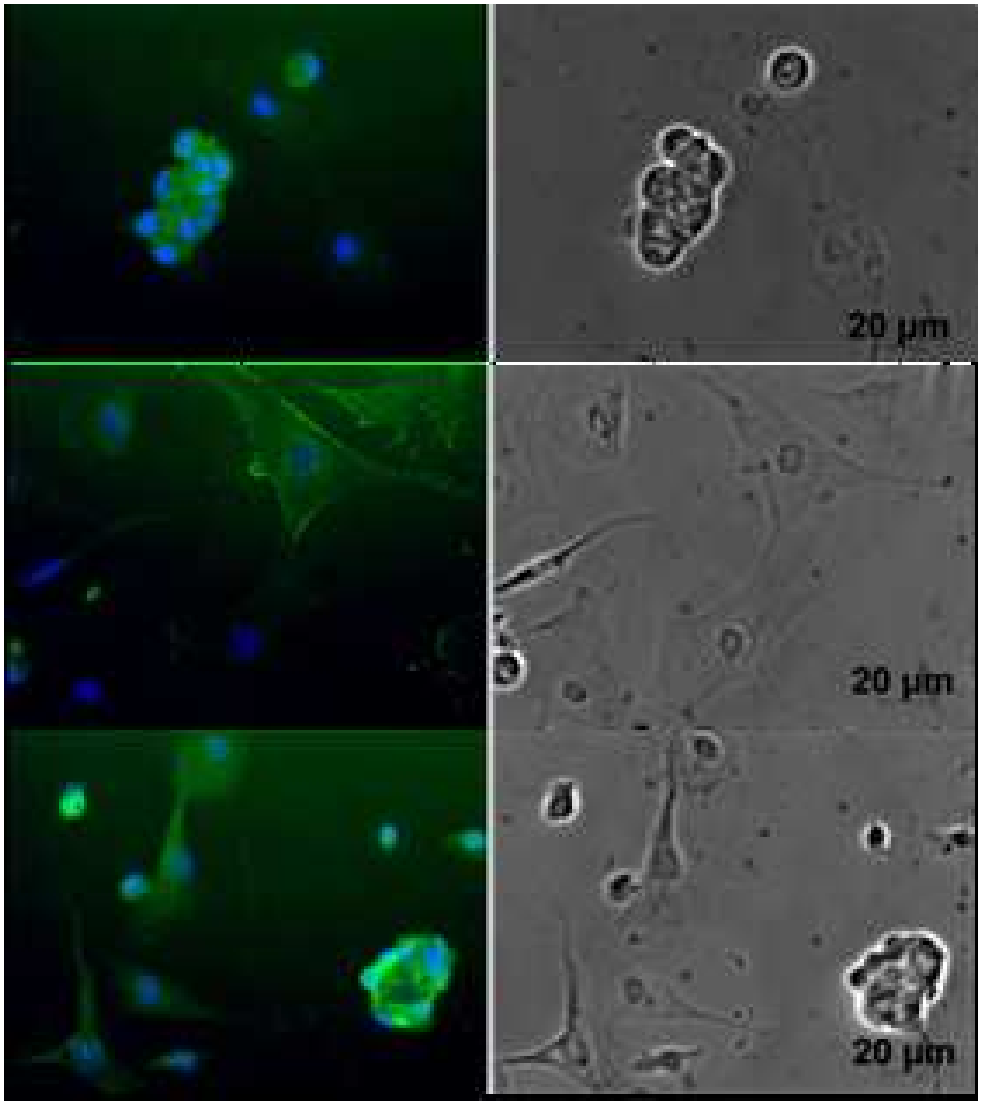


Figure 4.15. CSCs express c-Kit using IFA. C-Kit expression was located within cytoplasm and upon cell surface within adherent cells. Representative light microscope images are seen paired with IFA images. Negative controls were performed using secondary antibody only (Figure 4.16 B), showing DAPI only staining. Magnification x 400.

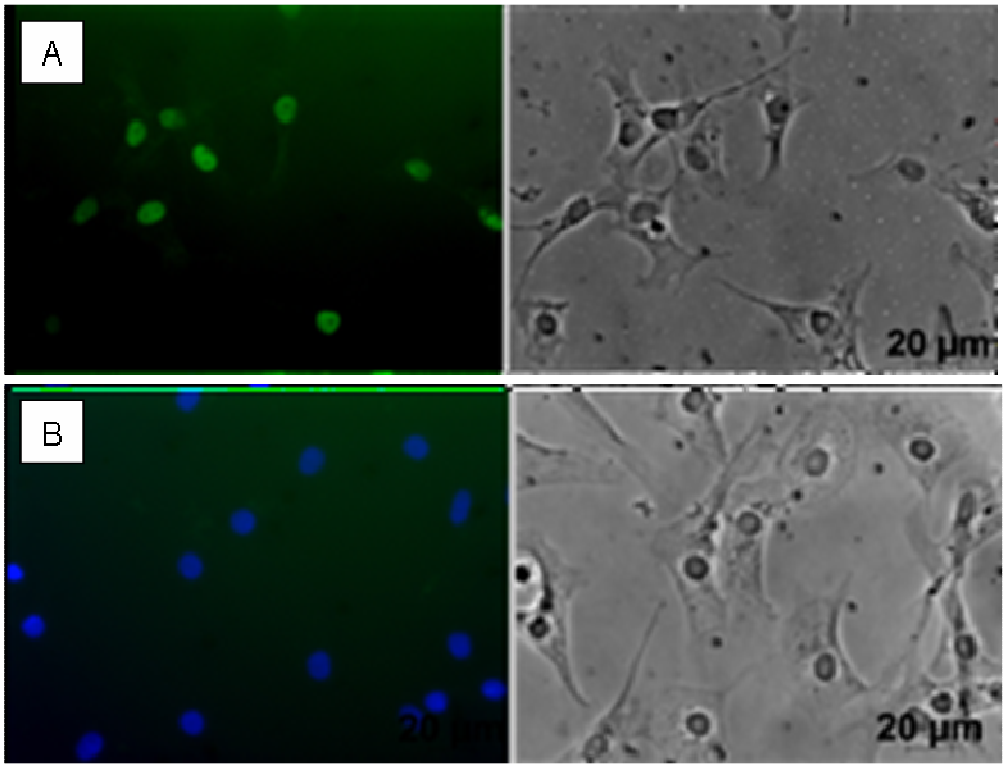


Figure 4.16. . CSCs express Flk-1 using IFA. Flk-1 expression was located within the nucleus of adherent cells (A). Representative light microscope images are seen paired with IFA images. Negative controls were performed using secondary antibody only (B), showing DAPI only staining. Magnification x 400.

Following this stem cells were again grown on gelatin coated chambered slides for 5 days to allow the growth of cardiospheres. These were labelled for c-Kit. Again negatives controls were run in conjunction as before (Figure 4.17).Cardiospheres were

found to be strongly positive for c-Kit expression, which was not seen in negative controls. DAPI labelling of nuclei clearly showed living cells within the spheres.

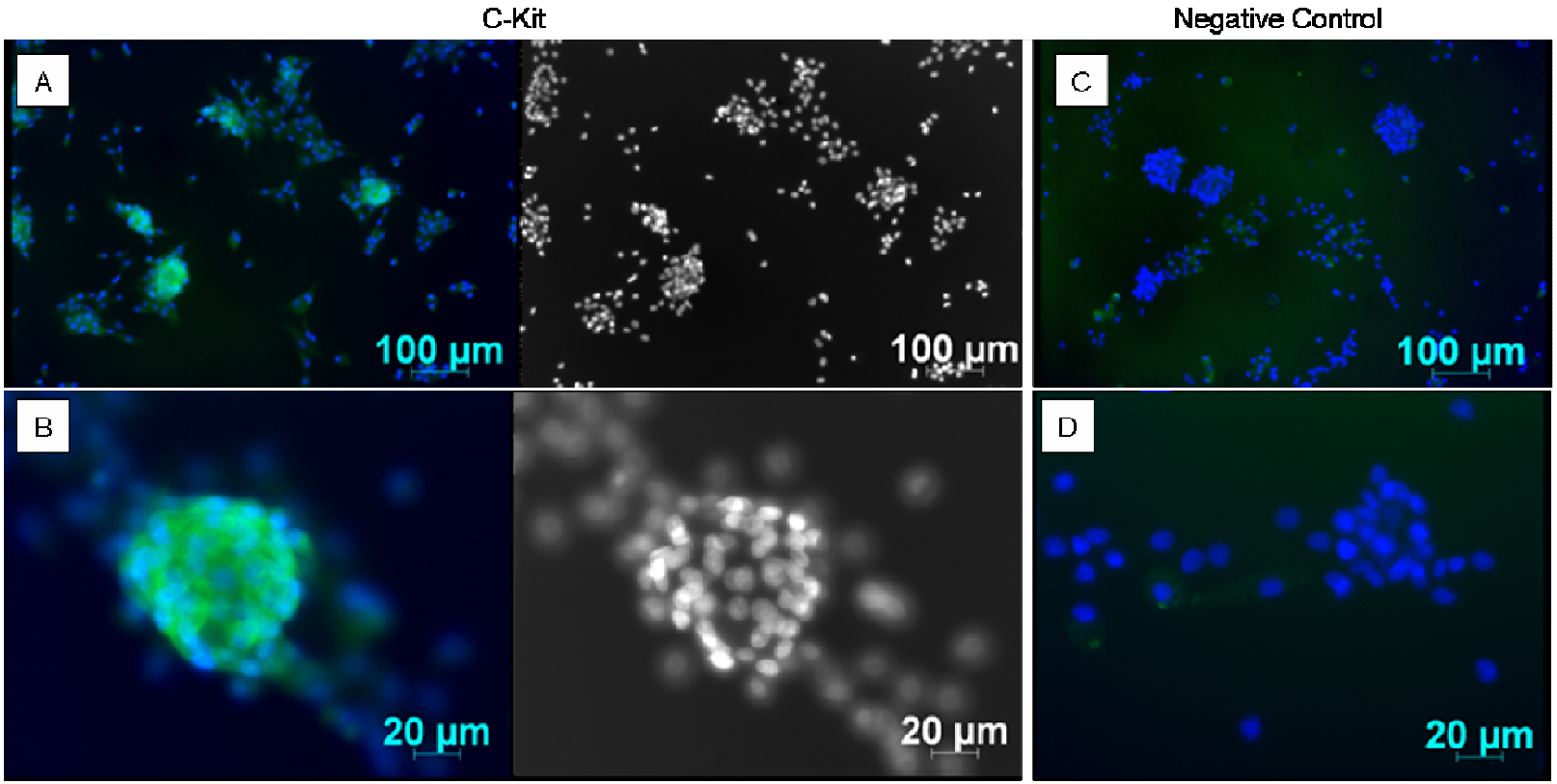


Figure 4.17. Cardiospheres were found to strongly express c-Kit. (A) Multiple cardiospheres can be seen at low magnification labeled clearly for c-Kit with paired corresponding grayscale image of DAPI stained nuclei (magnification x 100). At higher

magnification (B) the sphere cells can be seen brightly labeled for c-Kit (magnification x 400). Negative control spheres were labelled with secondary antibody only and clearly show no c-Kit labeling within spheres.

4.3.5 Magnetic Cell Sorting of Cardiac Stem Cells

Putative CSCs were partially selected for stem cell phenotype by transfer into cardiosphere media for a minimum of 7 days. Following this CSCs were sorted for c-Kit using MACS. The c-Kit magnetic beads pulled out an approximately 2-4% positive fraction from the cell suspension, clearing debris and fibroblast cells from the initial cell suspension. These cells appeared morphologically similar to the putative cardiac stem cells at sorting and during post-sorting culture (Figure 4.18).

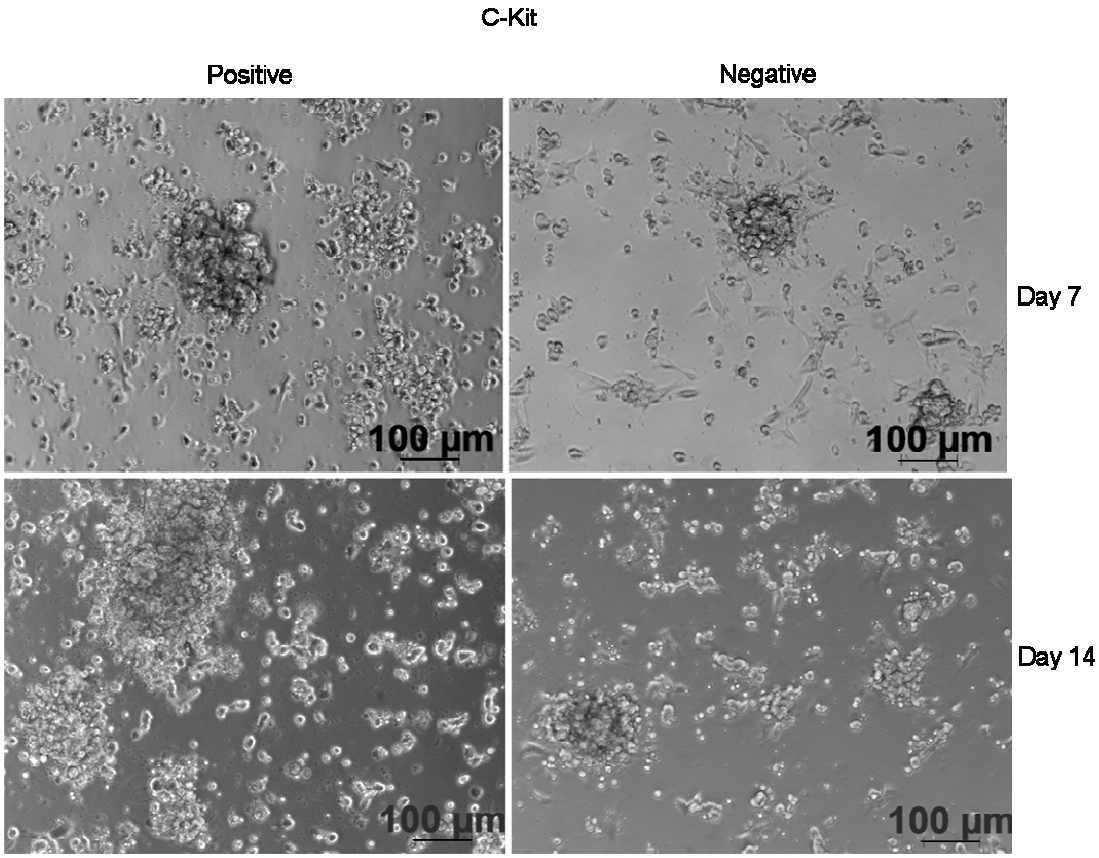


Figure 4.18. Culture morphology of c-Kit sorted cells at 7 and 14 days post sorting. Spheres and adherent cells formed in both c-Kit positive and negative populations at both time points. Magnification x 100

4.3.5.1 Transcriptional Analysis of MACS Sorted Cells

RT-PCR analysis was used to examine the expression of c-Kit, Flk-1 and islet 1 in cells immediately post-sorting. Three dogs were used, creating triplicate results. No difference was seen in the expression of c-Kit across the positive and negative fractions suggesting poor selection efficiency. Furthermore no difference was seen in the expression of islet 1 and Flk-1. D17 adherent cell RNA and whole heart tissue RNA were run as positive controls, and GAPDH was multiplexed as an internal positive control (Figure 4.19).

C-Kit sorted cells were then placed into CSC culture conditions in cardiosphere media for 14 days to examine any expression changes over time. Two dogs were used for this study. No difference in c-Kit expression was seen between positive and negative sorted fractions at time 0, 7 or 14 days using semi-quantitative RT-PCR (Figure 4.20). GAPDH was run as a multiplexed positive control.

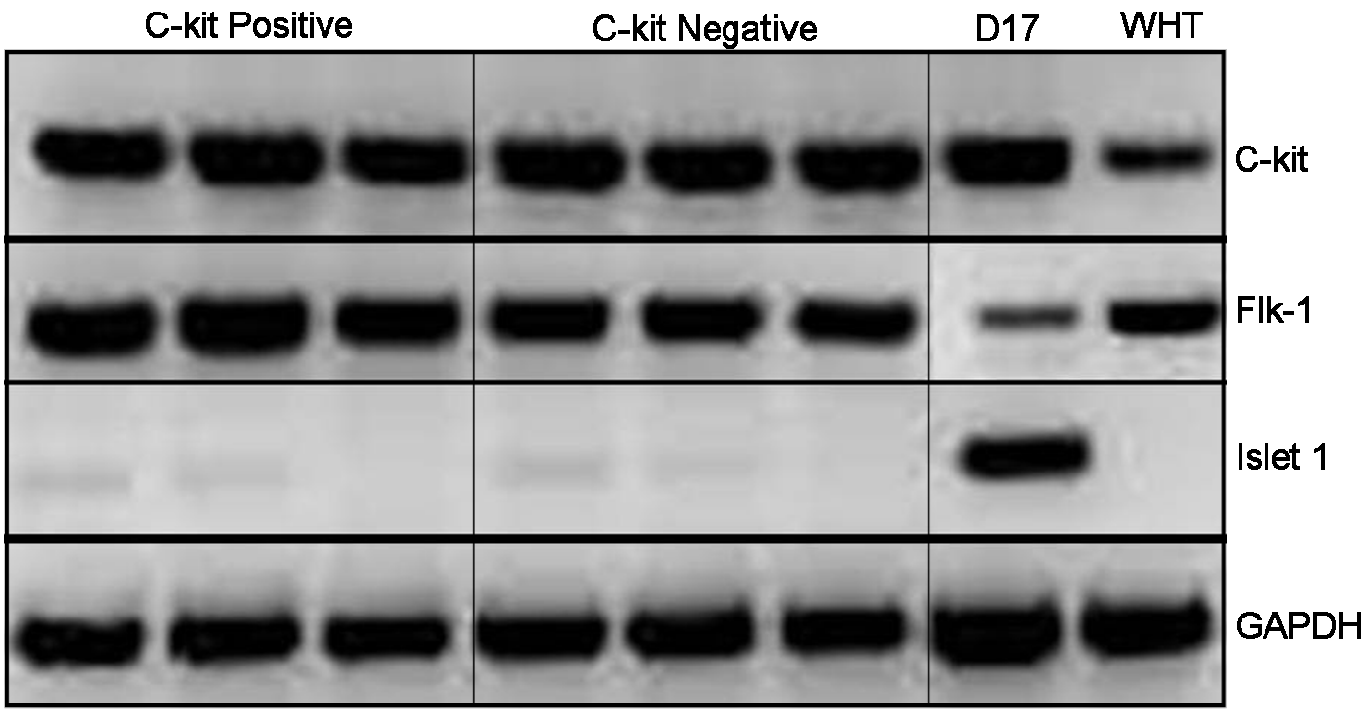


Figure 4.19. Semi-quantitative RT-PCR analysis of c-Kit sorted cells. Expression of c-Kit, Flk-1 and Islet 1 was compared in positive and negative fractions in triplicate, using D17 and whole heart tissue (WHT) RNA as positive controls, and GAPDH run as a multiplex control.

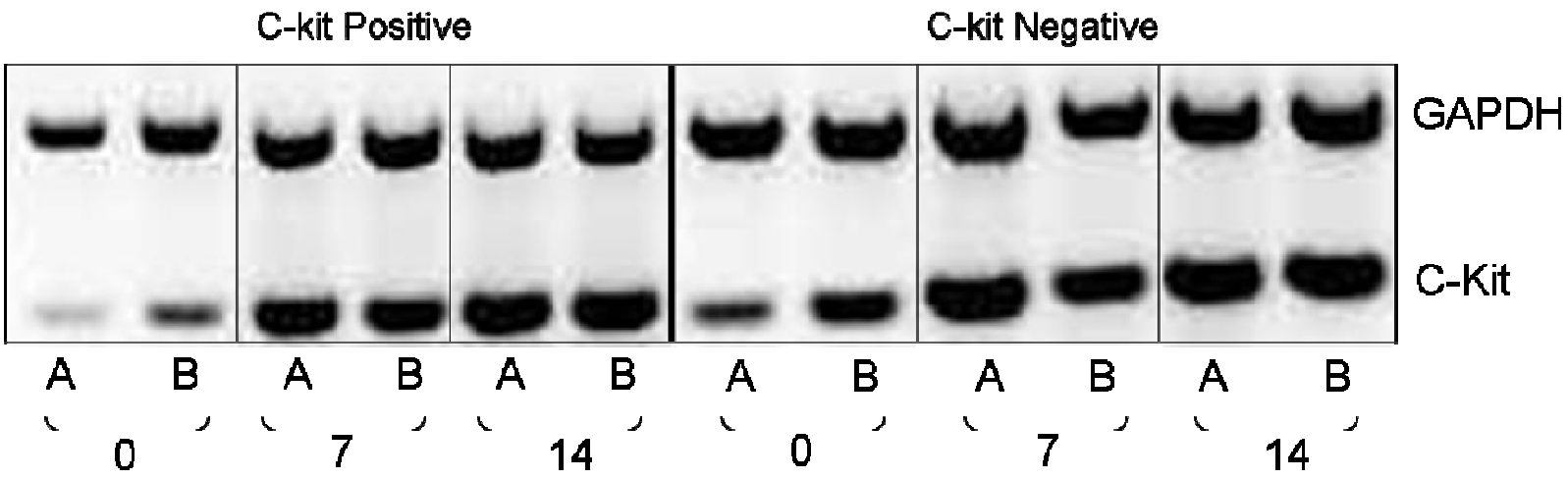


Figure 4.20. Semi-quantitative RT-PCR analysis of c-Kit sorted cells over time in culture. Once sorted cells were cultured for 14 days, and RNA extracted as days 0, 7 and 14. Samples were run with duplicate dogs (A and B). GAPDH was run as a multiplex control.

4.3.5.2 Immunofluorescence following MACS of Cardiosphere Cultures

Protein expression of Oct 4, Nanog, STAT 3 and c-Kit protein expression was investigated following MACS sorting for c-Kit of CSCs which had been in cardiosphere media for 16 days.This was examined in both positive and negative fractions. There was an approximately 2-4% pull down of c-Kit positive cells from the whole cardiosphere preparation, comparable with previous sorting. Labelling for Oct 4, Nanog, STAT 3 and c-Kit was poor, with low fluorescent signal seen in cytoplasmic locations in all cells examined (data not shown).

4.3.6 Differentiation of Cardiac Stem Cells using Published Protocols

4.3.6.1 Cell Morphology during Differentiation

CSCs were plated onto adherent gelatin cultures and exposed to four alternative differentiation protocols; two standard and two modified (see Materials and Methods above) in triplicate wells. A negative control culture of CSCs maintained in stem cell media was run concurrently, and formed cardiospheres as previously described and maintained these over three weeks. In all the wells following the four protocols (Figure 4.21) the CSCs initially formed a flattened extending morphology, with evidence of cell replication with increasing confluence. Over the period of the experiment the cells continued to form confluent flattened layers, but in the protocols aimed specifically toward differentiation (Smits protocol with TGFβ and Oh protocol with 5'AZA) there

appeared to be lining up and organisation of the cells, when compared to the modified protocols aimed at limited or no differentiation (Smits protocol excluding TGFβ and Oh protocol excluding 5'AZA).

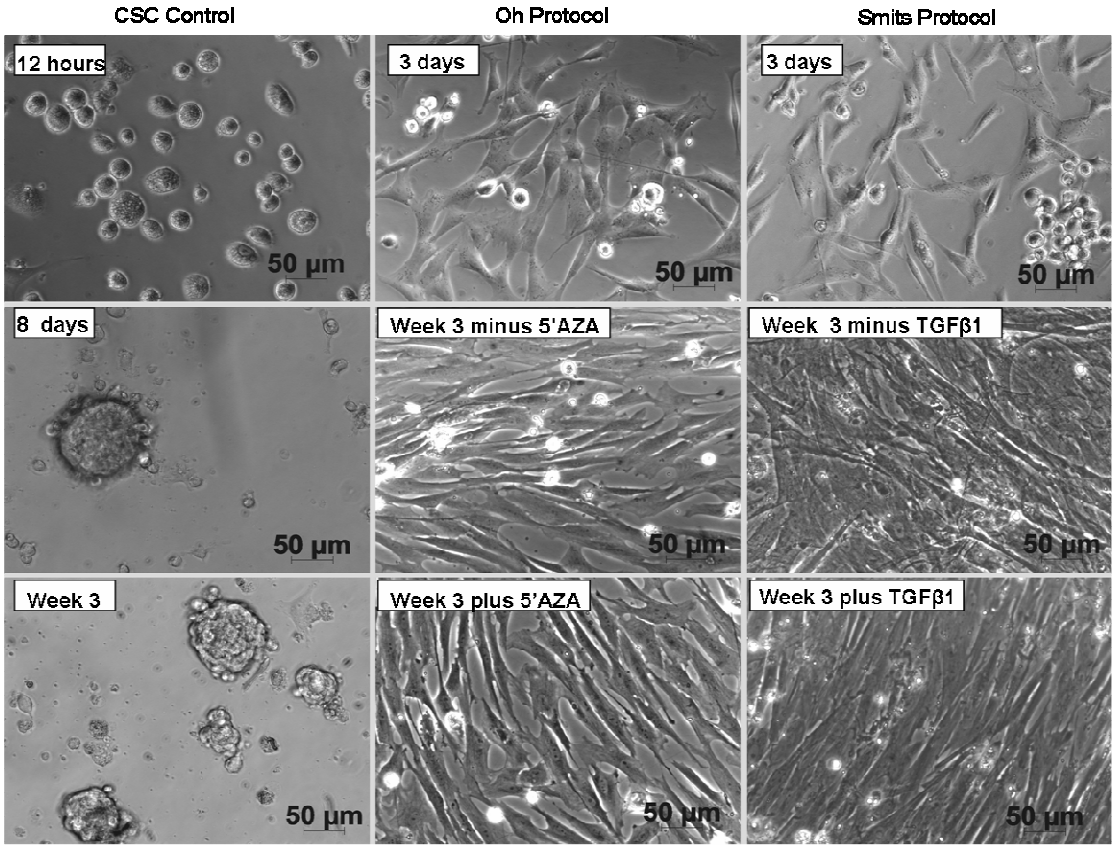


Figure 4.21. Morphology of CSCs during cardiac directed differentiation. CSCs kept in serum free cardiosphere media showed round phase bright morphology after 12 hours, which then developed into cardiosphere formation seen at 8 days and maintained for 3 weeks. CSCs cultured using the Oh and Smits protocol both showed flattened elongated morphology after 3 days in culture. Morphology of CSCs after 3 weeks in each protocol and modified protocols show some morphological alteration. Magnification x 200.

4.3.6.2 Marker Expression Patterns of Differentiated Cardiac Stem Cells

Following the differentiation experiment, triplicate wells were pooled and RNA extracted. Using semi-quantitative RT-PCR, gene expression profiles for each protocol were analysed and compared to a standard CSC and whole heart expression pattern (Figure 4.22). CSCs following the differentiating and modified Smits protocols appeared to have similar expression patterns; c-Kit, Nkx2.5, vWF and CTT had low expression compared to WHT whereas GATA 4, Flk-1, VEGF had high expression. SMA and glomulin showed similar expression levels to WHT, and there was no expression of CTI, the ryanodine receptor and the β_1-adrenergic receptor. Those following the Oh differentiating and modified protocols suggested more striking differences in expression levels. Specifically for the Oh protocols; c-Kit appeared to have lower levels of expression in the differentiated cells compared to the modified protocol; Nkx2.5 appeared upregulated in the differentiated cells; GATA 4 expression remained high following both protocols; no islet 1 expression was seen; Flk-1 expression seemed to decrease in differentiated wells compared to undifferentiated; SMA and glomulin expression seemed to remain at high levels across both Oh protocols; CTT appeared upregulated in differentiated wells, particularly for dog B compared to the modified protocol. VEGF displayed multiple bands corresponding to splice variants. In all four protocols no expression of the cardiac functional protein genes CTI, cardiac ryanodine receptor and the β_1-adrenergic receptor was seen.

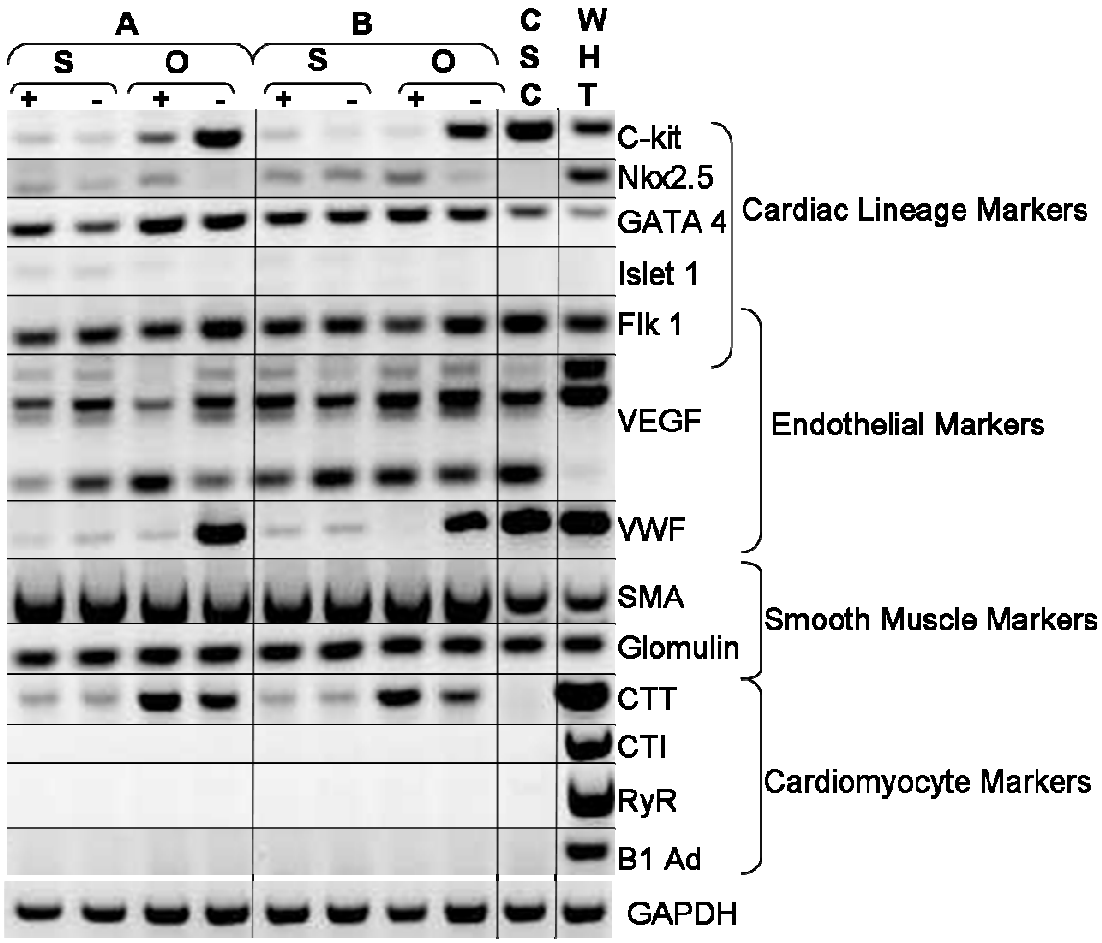
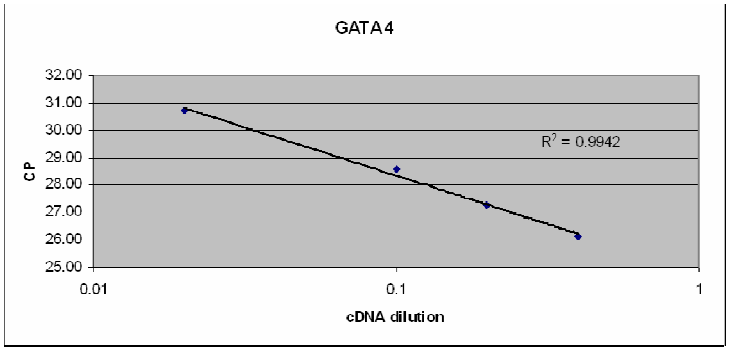
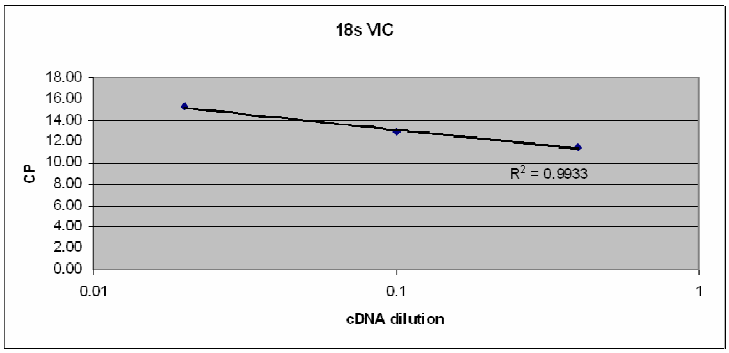


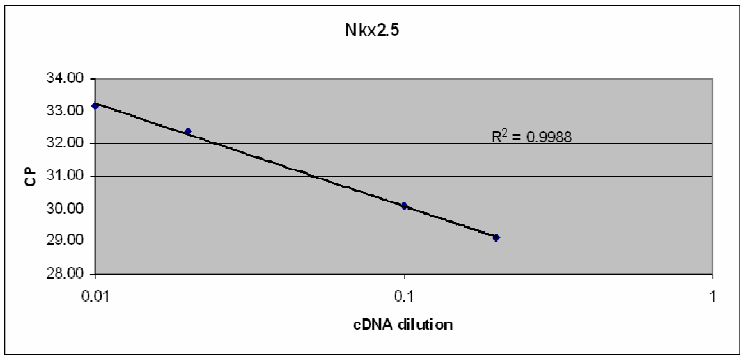
Figure 4.22. Semi-quantitative RT-PCR analysis of marker expression following differentiation using Oh standard and modified protocols (O + and -) and Smits standard and modified protocols (S + and -) for two dogs, A and B. Control samples of cardiac stem cells (CSCs) and whole heart tissue (WHT) were run as controls representing pre-differentiation and complete differentiation respectively. VEGF–vascular endothelial growth factor; VWF–von Willebrands Factor; SMA–smooth muscle actin; CTT-cardiac troponin T; CTI–cardiac troponin I; RyR–cardiac ryanodine receptor; B1 Ad-β_1-adrenergic receptor. GAPDH was run as a multiplexed in house control gene.

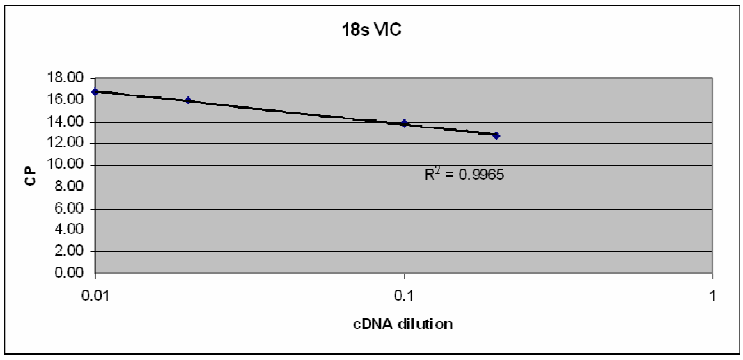
4.3.6.3 Quantitative RT-PCR Analysis of Oh Protocols

Following the semi-quantitative RT-PCR analyses of the CSC differentiation experiment it was indicated that the Oh differentiation protocol generated a stronger cardiac differentiation effect than the Smits protocol. Therefore four key markers of interest were selected to be analysed quantitatively, to ensure that suggested differences seen on gel analysis corresponded to real differences in expression levels. Nkx2.5, Flk-1, GATA 4 and CTT expression were examined using quantitative RT-PCR. All experiments were standardised using 18s ribosomal RNA, and compared directly to whole heart expression levels. Initial primer optimization was performed and generated R^2 values which were all above 0.99 in value (Figure 4.23).


GATA4
32.00
31.00
30.00
29.00
28.00
27.00
26.00
25.00
CP
R^2 = 0.9942
0.01
0.1
1
cDNA dilution


18s VIC
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
CP
R^2 = 0.9933
0.01
0.1
1
cDNA dilution


Nkx2.5
34.00
33.00
32.00
31.00
30.00
29.00
28.00
CP
R^2 = 0.9988
0.01
0.1
1
cDNA dilution


18s VIC
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
CP
R^2 = 0.9965
0.01
0.1
1
cDNA dilution

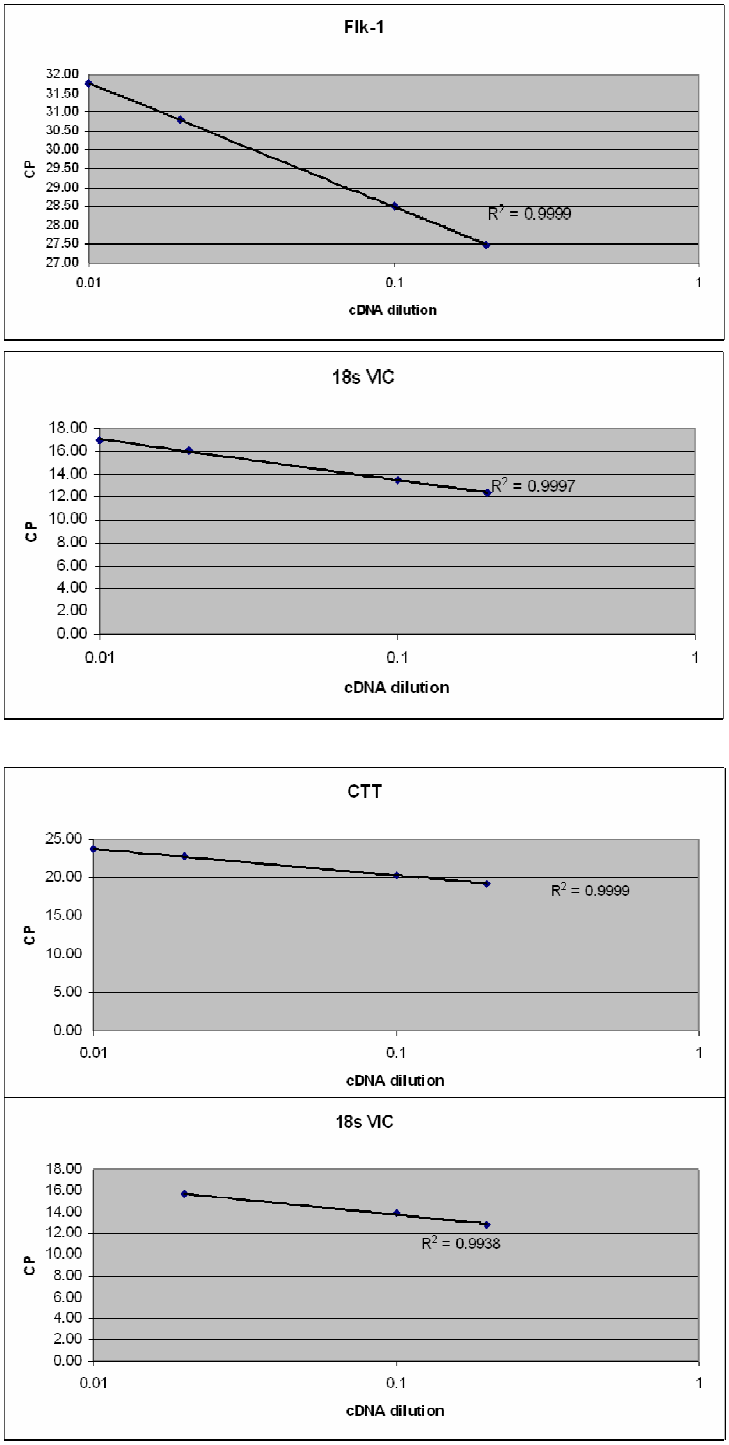


Figure 4.23. R^2 values for GATA 4, Nkx2.5, Flk-1 and CTT and their corresponding 18s rRNA VIC values following quantitative RT-PCR primer optimization.

Following primer optimization, gene expression analysis was performed for each marker for both Oh differentiated and modified protocol samples from both dogs relative to day 0 CSC expression. A 3.4 fold, and 5.1 fold (dog A and B respectively) up-regulation in expression of Nkx2.5 was seen in the cells under the differentiated protocol compared to that using the modified protocol, however GATA 4 appeared unchanged between the two protocols for both dogs, with fold changes of 1.1 and 1.3 for dogs A and B respectively. CTT had an approximately 1.5 fold up-regulation in differentiated cells in dog A, and a 3.3 fold up-regulation in dog B. Flk-1 was seen to be at lower expression in differentiated cells, with a 6.4 fold down-regulation for dog A, and 3.2 fold down-regulation for dog B between differentiated and undifferentiated protocols (Figure 4.24).

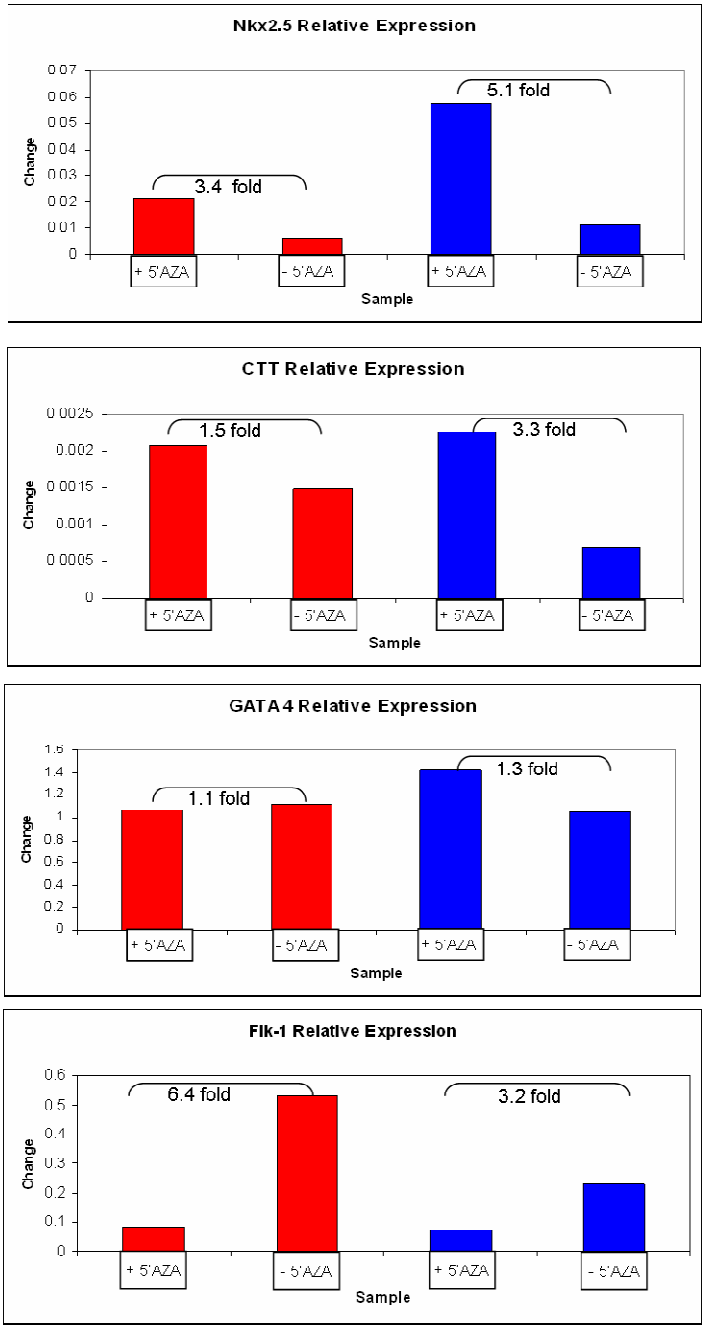
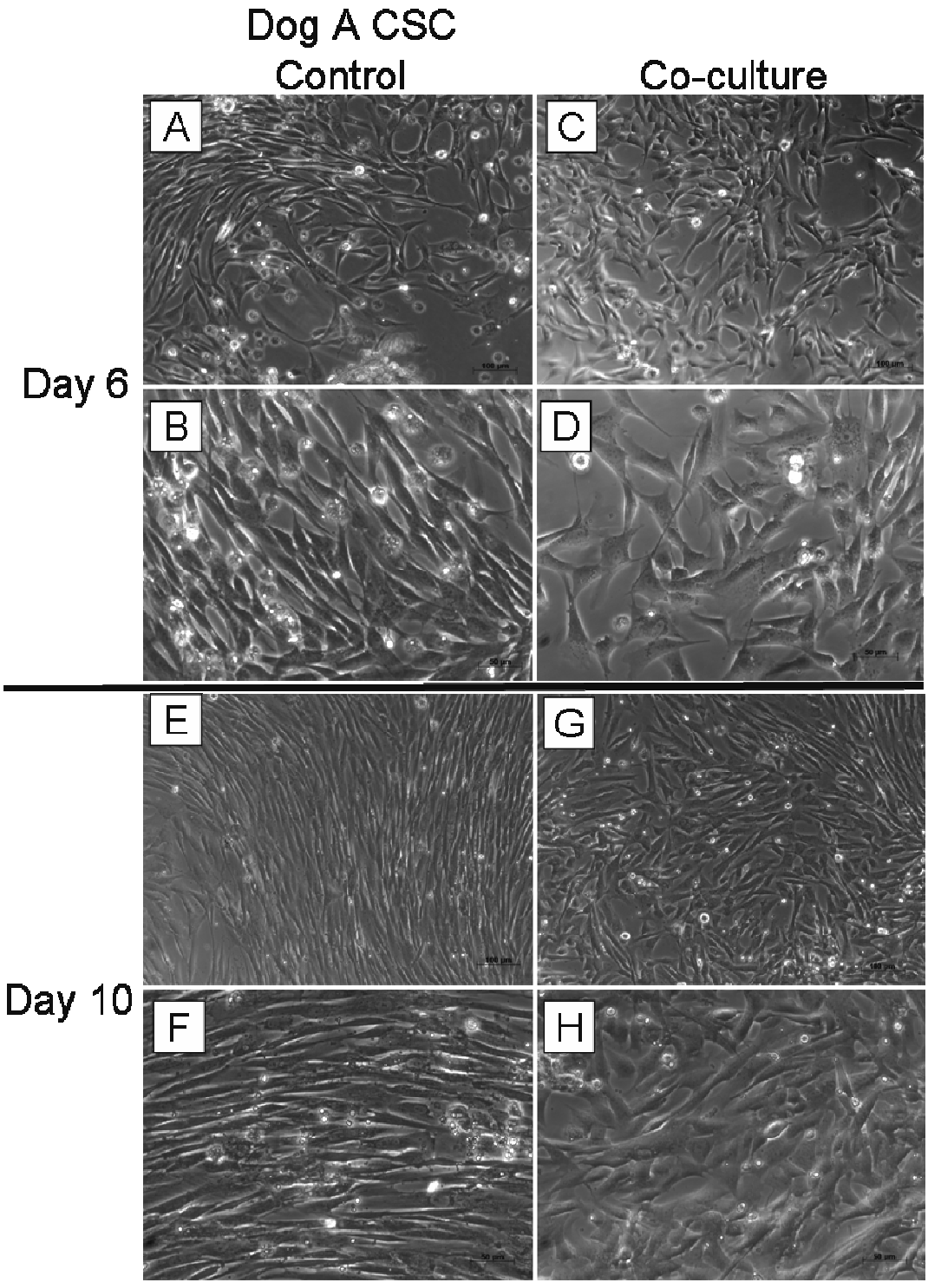


Figure 4.24. Quantitative RT-PCR analysis of marker expression from two dogs, A (red) and B (blue) following Oh protocol cardiac directed differentiation. Fold change was recorded relative to day 0 expression. Differentiation with and without 5'AZA is compared for four markers of interest, GATA 4, Nkx2.5, CTT and Flk-1.

4.3.7 Co-Culture of Cardiac Stem Cells with Mesenchymal Stem Cells

4.3.7.1 Cellular Morphology during Differentiation

Following plating of CSCs onto gelatin in transwell co-culture plates, the cells became adherent and assumed a more flattened morphology. Cells increased in number over time in culture, and morphologically there was a suggestion of a more organised pattern of cells in the control wells compared to the co-cultured wells in both dogs A and B, which became particularly evident at day 10. In co-cultured wells by day 10 there appeared to be a cellular morphological change with cells starting to assume a more cobblestone appearance particularly for dog B (Figure 4.25).


Dog A CSC
Control
Co-culture
A
C
Day 6
B
D
E
G
Day 10
F
H

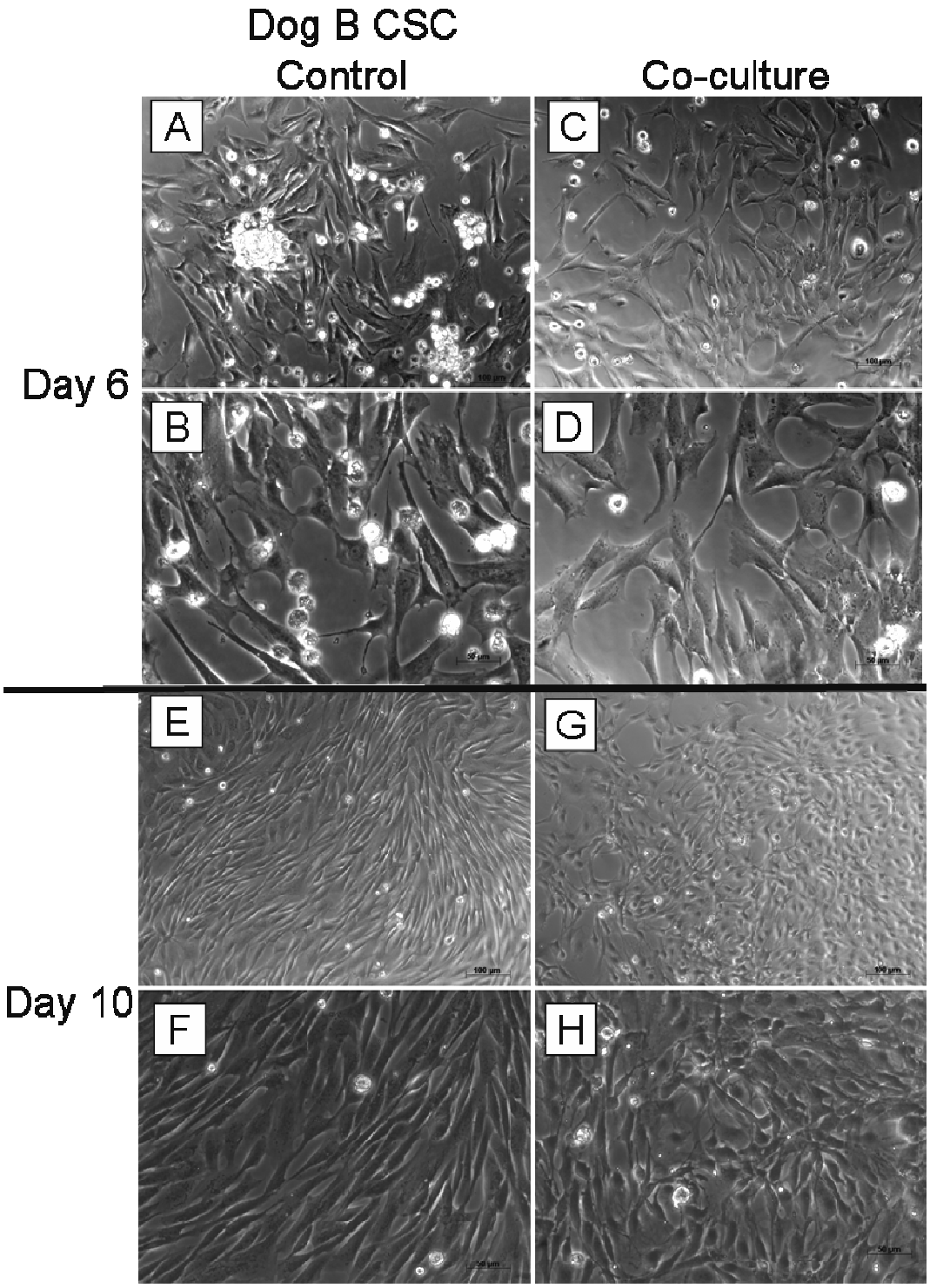

Dog B CSC
Control
Co-culture
A
C
Day 6
B
D
E
G
Day 10
F
H

Figure 4.25. Cellular morphology during co-culture of canine CSCs with autologous MSCs. Canine CSCs demonstrated morphological changes during co-culture compared to control wells at both time points for dogs A and B (Magnification A, C, E, G x 100; B, D, F, H x 200).

4.3.7.2 Transcriptional Analysis of Cardiac Stem Cells during Indirect Co-Culture

Gene expression analysis was performed on pooled triplicate RNA from co-culture and control wells using semi-quantitative RT-PCR (Figure 4.26). CD44 and CD34 (MSC markers) expression was seen in both dogs at high levels from day 0 through to day 10. Furthermore, there was a suggestion of increased expression of CD34 in co-cultured wells compared to control wells at both time points. Dog A had high levels of CD45 expression at day 0 which decreased to lower levels at day 6, and even less by day 10. Dog B also had high CD45 expression at day 0, which remained high at day 6 for both control and co-cultured wells, but then decreased in co-cultured wells at day 10, whereas control wells remained high. Dog A had low levels of c-Kit expression at day 0, which disappeared completely at day 6, and returned with faint expression at 10, with a slightly higher level in control wells compared to co-cultured. Dog B had a higher level of c-Kit expression at day 0 than dog A, but again all expression disappeared by day 6 in all wells, and only day 10 co-cultured wells showed very low level expression. Both dog A and B demonstrated expression of GATA 4 at day 0, which appeared to increase in all wells at day 6, and then increase again in all wells by day 10. Both dog A and B showed

no expression of Nkx2.5 at day 0, which is then faintly expressed in all wells at day 6 and then expressed at higher levels by day 10 with slightly higher expression in control wells compared to co-cultured. Flk-1 expression was relatively low at day 0, with slightly higher expression seen for dog B than dog A. Expression appeared to increase dramatically in co-cultured wells at day 6 compared to controls in both dogs, and then by day 10 expression was higher in all wells, but again appeared slightly higher in co-cultured wells than control wells. VEGF expression demonstrated multiple banding representative of splice variation in both dogs. Expression appeared to be relatively steady throughout the experiment. Both dogs expressed vWF at day 0, which was then switched off at day 6 in control wells, whereas in co-culture wells expression remained high. In dog A expression was present in controls wells at day 10, with higher expression in co-culture wells. In dog B expression is not seen in control wells at day 20, with higher expression seen in co-culture wells. SMA was expressed in both dogs at a steady level throughout the experiment. Glomulin expression levels appeared to be highest at day 10 in all samples in both dogs, compared to day 0 and 6. Connexin 43 (CNXN 43) was expressed at steady levels at all time points in dog A, whereas in dog B there appeared to be slightly lower expression at day 0, but then high expression is maintained in all samples at all time points thereafter. Islet 1, β_1-adrenergic receptor, CTI, RyR, CTT were not expressed in any samples (data not shown).

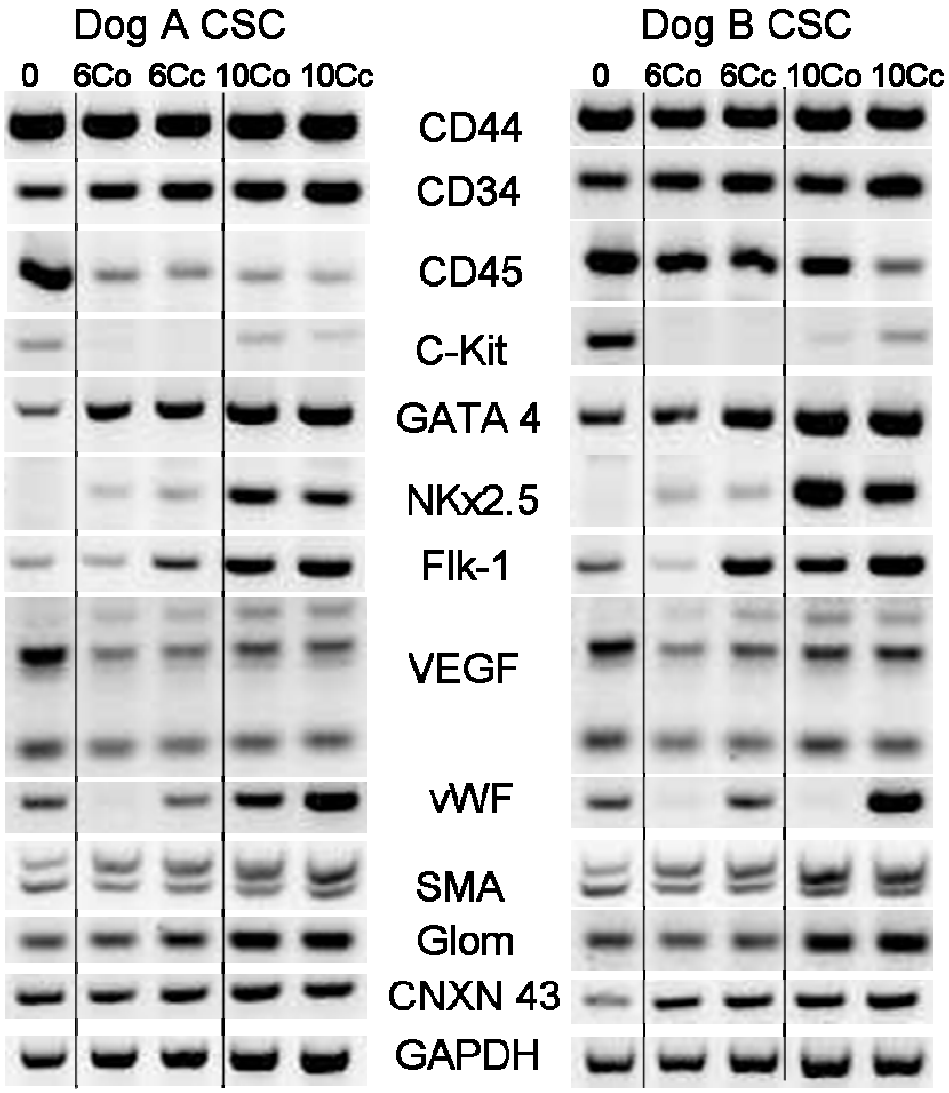


Figure 4.26. Semi-quantitative RT-PCR analysis of CSC co-culture expression changes. CSCs from two dogs, A and B, were co-cultured in a transwell system with autologous MSCs and were subsequently analysed at three time points, day 0, 6 and 10. Control wells (Co) were run concurrently with co-culture wells (Cc) at each time point. VEGF-vascular endothelial growth factor; vWF–von Willebrands Factor; SMA–smooth muscle actin; Glom–Glomulin; CNXN43–Connexin 43. GAPDH was run as a multiplexed in house positive control gene.

4.4 Discussion

4.4.1 Can Adult Cardiac Stem Cells be Isolated and Cultured from the Dog?

In this study we have isolated cells from the canine heart, which demonstrate phenotypic and genotypic characteristics consistent with an adult stem cell. The culture technique that this study employed proved reliable in reproducing directly comparative cardiosphere populations from multiple dogs of different breeds, ages and sex. In addition, our cell populations behaved in a similar way to that described in the literature for a number of other species including humans (Messina et al., 2004, Smith et al., 2007).

An interesting observation made at the start of the study was that sections of atrial tissue explants produced greater populations of stem cells compared to sections of similar size harvested from ventricular tissue. This finding was so consistent that we subsequently confined our harvests to atrial tissue. This observation is supported by the literature with descriptions of stem cell niche locations in other species, demonstrating that although stem cell niches in the adult heart are dispersed throughout the myocardium, the number of niches are typically higher in the atrial and apical myocardium compared to the base mid-region of the heart (Urbanek et al., 2006).

The canine CSC population was phenotypically similar to other stem cell populations, capable of being maintained over extended periods of time in serum free conditions. In

addition cells were capable of clonal expansion, a critical characteristic of stem cells. Following low-density plating, large dense clusters of cells grew out from single cells. On subsequent passage it was observed that there was a large central cell, surrounded by cells of varying size. This could reflect both asymmetric division, another key characteristic of stem cells, and also that the cells were of different ages, suggesting the original stem cell was generating daughter cells over time, and not in a single division.

4.4.2 Do Canine Cardiac Stem Cells Express Stem Cell Markers?

From an expression profile perspective, canine CSCs expressed certain markers that would be consistent with other stem cell types and in addition did not express lineage markers such as cardiac structural proteins or contractile proteins. We firstly investigated the expression of stem cell pluripotency markers.

4.4.2.1 Oct 4, Nanog, STAT 3

Expression of three classical stem cell markers, Oct 4, Nanog and STAT 3 was examined using a combination of RT-PCR, IFA and western blotting. Oct 4 expression was demonstrated in cardiosphere culture using IFA, and showed expression in cytoplasmic locations. Given the non-specific banding results from the western blot, the positive IFA result must be interpreted with caution. Oct 4 expression has been described in adult stem cells (Beltrami et al., 2007), however Oct 4 is known to have multiple splice variants, including Oct 4A, Oct 4B, and Oct 4B1 (Takeda et al., 1992,

Atlasi et al., 2008). The location of each of these variants is different, with Oct 4A predominantly nuclear and Oct 4B cytoplasmic, and Oct 4B1 found in both locations (Wang and Dai, 2010). Expression of the different variants alters with cell source; Oct 4A and Oct 4 B1 are expressed primarily in ES cells and are therefore reliable markers for stemness. However, Oct 4B is expressed in somatic cells, and therefore cannot be used as a stem cell marker (Atlasi et al., 2008). To complicate the field further, Oct 4B1 is capable of alternative splicing and re-forming Oct 4B, generating three alternative protein isoforms (Gao et al., 2010). Therefore, the positive result using cardiospheres may represent a non-stem cell variant of Oct 4 expression.

Nanog expression was seen using IFA in CSCs, but was found to have non-specific banding using western blot, and no transcript identified using RT-PCR. The discrepancy in expression is difficult to explain. An alternative splice variant of Nanog has been described, which showed increased expression within adult stem cells compared to ES cells, however both forms of Nanog were found to act in a similar fashion and therefore the relevance of the new variant is questionable (Kim et al., 2005b). Nanog location is generally considered to be nuclear, and functions as a nuclear transcription factor driving self-renewal (Chambers et al., 2003). The cytoplasmic staining seen in our cells using IFA, in combination with the negative RT-PCR results may imply non-specific binding of the Nanog antibody in canine adult stem cells.

STAT 3 was expressed using RT-PCR and western blot in CSCs at high levels. STAT 3 functions in conjunction with Nanog to maintain self-renewal, however given the

spurious Nanog results it would be difficult to make the assumption of this factor acting in that way in canine CSCs (Chambers et al., 2003). STAT 3 is not stem cell specific, and has been described in somatic cells. Furthermore STAT 3 is now found to be highly associated with oncogenic transformation; persistent activation of STAT 3 leads to a more aggressive cancer cell type, and poorer prognosis (Hirano et al., 2000, Yu et al., 2009a). Expression of STAT 3 in the heart is thought to have a cardioprotective property, and is upregulated in response to stress signals (Boengler et al., 2008) and STAT 3 expression is upregulated via the CXCR4 pathway in response to ischemia (Huang et al.). STAT 3 translocates to the mitochondria and protects against ischemia via induction of oxidative phosphorylation (Wegrzyn et al., 2009, Qiu et al., 2011). STAT 3 is therefore a critical factor expressed within cardiomyocytes and it could be assumed therefore that expression would be expected within the CSC population, reflected by our results.

Oct 4, Nanog and STAT 3 were initially chosen due to their common use as pluripotency markers in stem cells. However, based upon the results obtained the breadth of markers was widened to include stem cell markers more specific to adult stem cells, and in particular those from the heart. As can be seen from this data, there are significant limitations in using antibodies for gene marker expression analysis, given the shortage of canine specific antibodies. This highlights the need for several modes of analysis when comparing gene expression.

4.4.2.2 Cardiac Stem Cell Markers

The CSCs were subsequently analysed for their expression of published CSC markers. Canine CSCs were c-Kit positive. As previously discussed c-Kit has been used to define and isolate CSCs from a number of species especially humans and c-Kit positive stem cells are capable of differentiating into cardiomyocytes (Beltrami et al., 2003, Goumans et al., 2007, Kubo et al., 2008, Tallini et al., 2009).

The canine CSCs also expressed Flk-1 at both RNA and protein level. Flk-1 analysis of early harvest stem cells using immunofluorescence confirmed the presence of the protein at a nuclear location, indicating an active form. Flk-1 has been shown to be expressed in some, but not all CSC populations. Bearzi *et al* described a human cardiac stem cell population positive for c-Kit, and negative for Flk-1 (Bearzi et al., 2007). In a subsequent study, the same author also described a c-Kit positive, Flk-1 positive population which behaved as a coronary vascular progenitor cell (Bearzi et al., 2009). Yang *et al* isolated a Flk-1 positive, c-Kit negative stem cell population from the human heart which gave rise to colonies which contained all three cardiac lineages (Yang et al., 2008). This interesting variation in c-Kit and Flk-1 expression may represent a heterogeneous stem cell population or may represent cells at different stages of stemness or differentiation.

In this study CSCs and whole heart demonstrated high levels of GATA 4 expression. This correlates with results seen by Goumans *et al* where whole human heart tissue had

similar expression profiles to their stem cell populations (Goumans et al., 2007). GATA 4 has been shown to be repressed by the expression of Nanog; during our experiments we found no reliable expression of Nanog and a high level of GATA 4 expression, which correlates with this data (Kim et al., 2005b). GATA 4 is one of the earliest transcription factors expressed in the developing heart, and is expressed abundantly in CSCs and cardiac myocytes during their lifespan. GATA 4 is thought to have cardioprotective properties, preventing post-infarction remodelling (Heikinheimo et al., 1994, Matsuura et al., 2004, Laugwitz et al., 2005, Beltrami et al., 2007, Goumans et al., 2007, Rysa et al., 2010); it is therefore unsurprising that it was expressed in both the whole heart controls, the stem cells and subsequently differentiated cell populations.

Nkx2.5 was expressed in whole heart tissue but surprisingly not in the canine CSCs. Nkx2.5 is a homeobox gene, is expressed early as part of the cardiac lineage (Martin et al., 2004) and has commonly but not exclusively been found to be expressed in CSCs (Beltrami et al., 2003, Laugwitz et al., 2005, Goumans et al., 2007). Oh *et al* isolated a population of murine cardiac stem cells using the mouse specific marker Sca 1 which were c-Kit and Nkx2.5 negative, and performed directed differentiation upon these cells which was then associated with an upregulation in Nkx2.5 and cardiac structural gene expression (Oh et al., 2003). Therefore the absence of Nkx2.5 expression may reinforce the primordial stem cell nature of our CSCs.

Canine CSCs were positive for the endothelial marker vWF. Interestingly, this has been proposed as an important finding in cardiac progenitor cells, which is not seen in other

stem cell populations such as mesenchymal stem cells and this result may reinforce the multipotent potential of our isolated cells, and in particular their potential for endothelial differentiation (Hu et al., 2003, Kattman et al., 2006, Welikson et al., 2007).

Islet 1 was not expressed consistently within the CSCs isolated. Interestingly, islet 1 positive cells were isolated twice, as could be seen in the CSCs culture expression analysis (Figure 4.14). Two separate dogs were positive for islet 1, dog 8 and dog 10 and these cells appeared to demonstrate a reduced expression of c-Kit and Flk-1. No other canine CSCs isolated were positive for islet 1. Interestingly, dog 10 was analysed at two time points, day 0 and day 6, and was only found to be positive only at day 6. This may mean that islet 1 is switched on in serum free culture, but given that no other CSCs demonstrated this then the cell may have a predetermined ability for islet 1 expression. Dog 8 was also found to express islet 1 at day 15, but due to the limitations of the experiment, no other time points were examined. We were unable to isolate CSCs specifically using islet 1 given its nuclear location therefore this interesting finding was unable to be analysed further. Islet 1 has been described as a cardiac stem cell marker, and labels cardiac progenitors from the secondary heart field (Cai et al., 2003). Traditionally these cells co-express Flk-1, and the reduced expression of Flk-1 seen in our stem cell population is therefore interesting. As previously described, c-Kit/Flk-1 variation in expression labels different subsets of cardiac stem cells, and our results may be a reflection of this heterogeneity. It may also be possible that the islet 1 cell populations are an earlier precursor to the c-Kit/ Flk-1 positive stem cells, and are found in lesser numbers, hence explaining our only occasional isolation.

Connexin 43 is used for cell-cell signalling and contact and is generally highly expressed within cardiac tissues (Dhein et al., 2002). Connexin 43 has been shown to increase following CSC differentiation (Messina et al., 2004). We found connexin 43 to be expressed in both CSCs and cells which had undergone differentiation. This limits the use of connexin 43 as a marker, and does concur with Gaetani *et al* who also found connexin 43 expression in CSCs before and after differentiation (Gaetani et al., 2009). However, the expression of connexin 43 in CSCs does imply a pre-determined functional ability for cell-cell signaling. Connexin 43 is commonly cited in the literature as a standard marker for cardiomyocyte differentiation. We investigated the expression of this marker not only in our cell populations and control tissues, but also found expression in several non-cardiac tissues. This is likely due to its nature as a gap junction protein and the fact that it can be found in several organ types, including uterus, ovaries and kidneys (Beyer et al., 1987).

In concurrence with CSC populations isolated from other species including humans, our cell population appeared to be CD45 negative (thereby not haematopoietic in origin) and lineage negative (Beltrami et al., 2003, Oh et al., 2003, Bearzi et al., 2007, Goumans et al., 2007), in that there was no evidence of expression of the cardiac functional genes CTT, CTI, RyR and the β_1-adrenergic receptor. This implies the primordial nature of these cells, given their direct isolation from cardiac tissue, and may support the suggestion that they are precursors of several cardiac cell types.

4.4.3 VEGF Splice Variation and Significance to Cardiac Stem Cells?

A further interesting finding was VEGF splice variation. A different variant pattern was seen between whole heart tissue and CSCs. VEGF is a major inducer of angiogenesis and may mediate the migration of CSCs via PI3K/Akt pathway (Tang et al., 2009). VEGF is known to have several isoforms, all described in humans; $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$ and $VEGF_{189,}$ $VEGF_{206}$. Furthermore, each VEGF splice variant binds to a specific receptor; $VEGF_{121}$ and $VEGF_{165}$ bind to Flt-1 whereas $VEGF_{121}$, $VEGF_{145}$ and $VEGF_{165}$ bind to Flk-1 (Neufeld et al., 1999). Each variant is thought to play a different role; $VEGF_{145}$ has been found to be the major isoform expressed in human reproductive carcinomas, $VEGF_{121}$ and $VEGF_{165}$ are thought to be involved in angiogenesis, and induce proliferation and $VEGF_{189}$ is thought to be involved in endothelial cell proliferation (Poltorak et al., 1997). $VEGF_{121}$, $VEGF_{145}$ and $VEGF_{165}$ are believed to be secreted forms, whereas $VEGF_{189}$ and $VEGF_{206}$ are cell surface bound and it has been found that human blastocysts differentially express variants during implantation (Krussel et al., 2001)

In animals VEGF variants are known as $VEGF_{120}$, $VEGF_{164,}$ $VEGF_{182}$ and $VEGF_{188}$; however $VEGF_{182}$ and $VEGF_{188}$ are usually impossible to separate due to only an 18bp difference in size. Multiple splice variants have been previously described in the dog and $VEGF_{164}$ has been found to be the dominant isoform in the canine heart, with $VEGF_{120}$ seen at low levels, and $VEGF_{182}$ and $VEGF_{188}$ seen as one band using RT-PCR (Jingjing et al., 2000, Bartosh et al., 2008, Uchida et al., 2008). No canine equivalent of

human $VEGF_{145}$ and $VEGF_{206}$ has been found even though canine and human VEGF share high amino acid homology (95%). Furthermore a significant increase in all VEGF isoforms in proportion to original levels was noted in canine hearts following ischemia (Jingjing et al., 2000). Based upon this information and our results it is most likely that $VEGF_{164}$ and the combined $VEGF_{182/188}$ are the dominant isoforms seen in whole heart tissue. Interestingly, in the CSC population these variants are also expressed, but a strong third band is seen which would correspond to the expression of $VEGF_{120}$ with a combined lower strength band corresponding to $VEGF_{182/188}$. This finding may be of great importance when isolating CSCs and could be used to determine stem cell type over differentiated cell types. The significance of the increased expression of $VEGF_{120}$ is yet to be discovered, but is an area of future research.

4.4.4 Can Canine Cardiac Stem Cells be Sorted using C-Kit?

C-Kit has commonly been used as a marker to sort putative CSCs from a mixed cardiac cell population (Beltrami et al., 2003, Kubo et al., 2008, Tallini et al., 2009). A magnetic cell sorting technique was utilised to sort a c-Kit positive and negative fraction from general explant cultures. This produced an approximate 2-4% positive cell fraction; this compares to Beltrami *et al*, which investigated lineage negative, c-Kit positive cells from whole rat heart, and found low numbers of positive cells at 1 cell in approximately 1×10^4 myocytes (Beltrami et al., 2003). The positive cell fraction displayed sphere forming properties, and was maintained in serum free culture in cardiosphere media for a further 13 days, before harvesting for RNA isolation. Semi-quantitative RT-PCR

analysis of c-Kit sorted cells at day of sorting demonstrated no obvious difference in c-Kit, Flk-1 and islet 1 expression between the positive and negative sorted fractions. Furthermore, sorted cells grown in stem cell culture showed no alteration in the expression of c-Kit over 14 days. This may suggest an inefficiency using the MACS technique, or may reflect the exponential nature of PCR amplification or a combination of the two. The sorted c-Kit populations were further analysed for the expression of Oct 4, Nanog, STAT 3 and c-Kit using IFA. Very poor labeling of the cells was seen with weak fluorescent signal in cytoplasmic locations for all four markers (data not shown). As previously discussed, Oct 4 and Nanog should be located in the nucleus, and c-Kit and STAT 3 can be cytoplasmic. The poor quality fluorescence meant that no result could be determined from this particular experiment. Again the technique was limiting, with very few cells available following sorting, which may have been due to cell death following sorting, therefore limiting the number of viable cells to grow on chambered slides. Furthermore as discussed previously there is a difficulty in obtaining canine specific antibodies making likelihood of poor specificity higher.

4.4.5 Are Canine Cardiac Stem Cells Capable of Cardiac Directed Differentiation?

4.4.5.1 Cardiac Differentiation using Published Techniques

CSC differentiation has been described using a variety of culture techniques and pharmacological interventions. Tallini *et al* differentiated murine cardiac c-Kit positive cells using bFGF (Tallini et al., 2009). Messina found that murine cardiospheres began spontaneous contractions, however human cardiospheres required co-culture with RNCM (Messina et al., 2004). Laugwitz also co-cultured murine islet 1 cells with RNCM to drive the differentiation process (Laugwitz et al., 2005, Moretti et al., 2006). Goumans *et al* differentiated human progenitor cells using 5'AZA followed by the addition of TGFβ1 and ascorbic acid (Goumans et al., 2007, Smits et al., 2009b) based on a technique described for murine ES cells (Behfar et al., 2002). Oh *et al* used 5'AZA and a serum concentration drop to trigger differentiation in murine adult stem cells (Oh et al., 2003). We selected two published techniques – Smits *et al* and Oh *et al,* and used standard differentiation protocols and modified protocols to assess differentiation. Under both standard differentiation protocols there were obvious phenotypic changes, with suggestion of cellular alignment, when cell populations were compared to the modified versions of each protocol and to control untreated cell populations. The alignment of cells observed in the standard protocols may represent development of an organised cell-cell contact, suggesting a more defined phenotype.

However, despite phenotypic changes in the standard Smits protocol versus the modified protocol excluding TGFβ1, there was very little difference between these cell populations at the level of transcriptional analysis over the range of markers analysed, suggesting that changes in gene expression observed were not associated with the exclusion of TGFβ1. Interestingly, both cell populations lost evidence of c-Kit and vWF expression, present in the untreated CSCs which may be a result of time and method of culture. There was also evidence of low levels of expression of CTT and Nkx2.5, which were not expressed in the untreated CSCs, which may have been an effect of TGFβ1. There was no evidence of expression of other structural proteins such as CTI, RyR and β_1-adrenergic receptor. This technique appeared to generate inefficient cardiac directed differentiation.

More striking changes were observed at the transcriptional level when comparing the standard Oh protocol versus the protocol excluding 5'AZA. This may suggest that 5'AZA and serum concentration are critical factors in altering expression profiles in canine stem cells. Following the standard Oh protocol, there was a loss of expression of c-Kit and vWF as for the Smits protocols. There was also expression of Nkx2.5 and CTT and unlike the Smits protocols expression levels were high. Cells treated following the modified Oh protocol also expressed CTT, but at a lower level than those under the standard protocol and did not lose c-Kit and vWF expression or exhibit Nkx2.5 expression. The smooth muscle markers SMA and glomulin were highly expressed in all

the cell populations. Again there was no evidence of expression of other structural proteins such as CTI, RyR and β_1-adrenergic receptor.

We therefore analysed the relative expression of the cardiac lineage markers Nkx2.5, GATA 4, Flk-1 and the cardiac differentiated marker CTT using quantitative RT-PCR following cells undergoing the standard and modified Oh protocols. There was upregulation in the expression of Nkx2.5 and CTT, and down regulation of Flk-1 in the cells following the standard protocol compared to those following the modified protocol which confirmed the findings seen in standard RT-PCR and reinforced the suggestion of cardiac directed differentiation, and potentially a move away from endothelial lineages. There was little difference seen in the expression levels of GATA 4 between the modified and differentiated protocols; GATA 4 expression is seen in both the cardiac stem cells and whole heart controls, which may suggest that maintenance of its expression is a requirement of these cells during the differentiation process.

Previous studies have used a variety of criteria to define cardiac differentiation. Spontaneous beating, not observed in our cells, is often used as a determinant of stem cell differentiation, and has been described in several papers; interestingly it is suggested that human cardiospheres require co-culture with RNCM to enable the initiation of beating and it may be that this technique is required for beating in canine cells (Matsuura et al., 2004, Messina et al., 2004, Beltrami et al., 2007, Goumans et al., 2007, Smith et al., 2007, Tallini et al., 2009).

In our study we utilised a comprehensive range of differentiation markers. Specifically in cells exposed to the standard Oh protocol, there was evidence of incomplete differentiation. This is based on phenotypic changes in the cells, the loss of the stem cell marker c-Kit, the loss of expression of vWF and the appearance of expression of both Nkx2.5 and the structural protein CTT. However other proteins involved in the contractile machinery of a differentiated cardiomyocyte were not expressed including CTI, RyR and the β_1-adrenergic receptor (Figure 4.27). We have therefore made the assumption that cardiac directed differentiation was achieved in part only, and that further investigation would be required to find the factor or factors required to trigger complete differentiation.

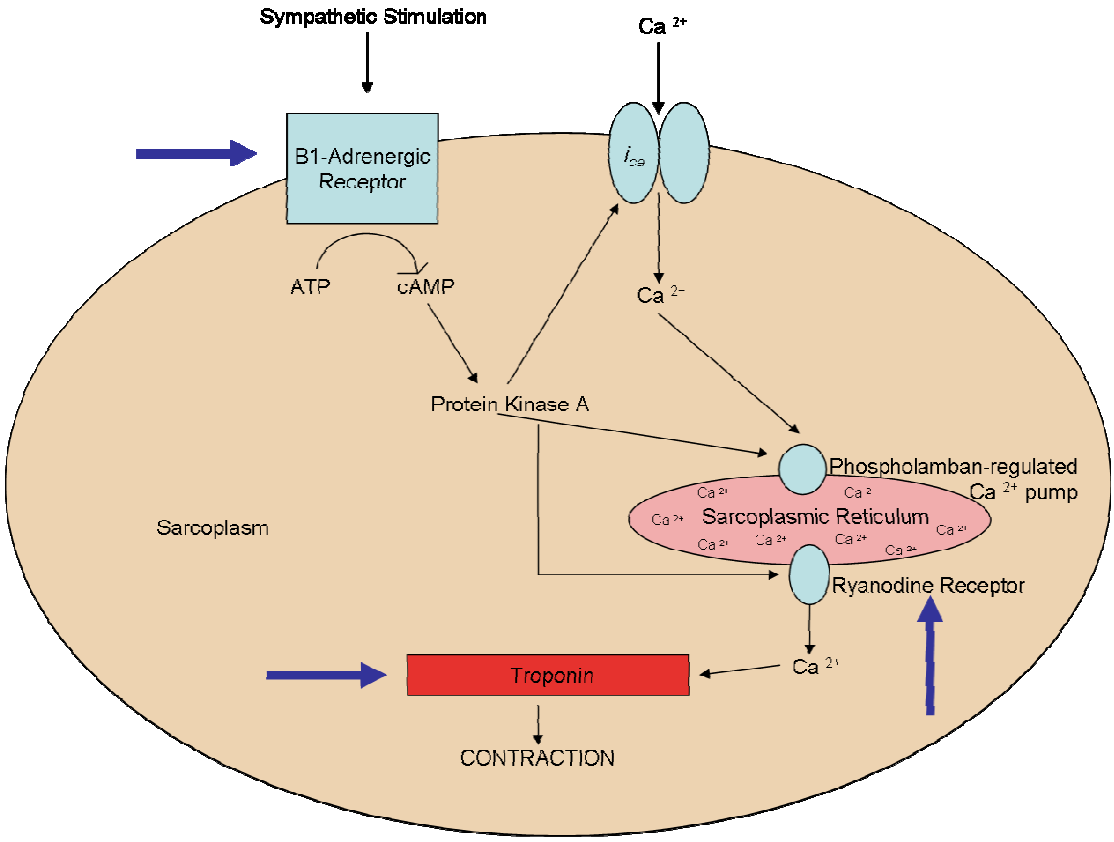


Figure 4.27. Cardiomyocyte functional proteins associated with cardiac contraction. Following stimulation of the β_1-adrenergic receptor with catecholamines such as noradrenaline, cyclic AMP (cAMP) is generated from ATP by the catalytic action of adenyl cyclase. cAMP activates the intracellular enzyme Protein Kinase A (PKA), which has several effects; a) PKA phosphorylates the calcium channels (i_{ca}) in the cell membrane increasing their open state, allowing more calcium influx into the cell and ultimately for stronger contractile force, b) PKA phosphorylates phospholamban, a regulatory protein for the inward calcium pump into the sarcoplasmic reticulum (SR), thus reducing phospholamban inhibition and removing free calcium into the SR, c)PKA

phosphorylates the ryanodine receptor, which acts in response to increased calcium by releasing further calcium into the cytosol of the cell. This released calcium from the SR binds to cardiac troponin C which is part of the troponin complex including C, T and I, which alters the position of the inhibiting tropomyosin molecule, exposing the actin sites and allowing for myosin to bind, and contraction to occur. Thus the β_1-adrenergic receptor, the ryanodine receptor, and cardiac troponin T and I are all critical elements in a functional cardiomyocyte (labeled with blue arrow) and were chosen as cardiac specific analysis genes.

4.4.5.2 Cardiac Differentiation using Indirect Co-Culture

Co-culture of different cell populations has previously been shown to drive differentiation. Co-culture of CSCs with RNCM has been shown to be an effective method to provide beating differentiated cardiac cells in humans, dogs and mice (Messina et al., 2004, Laugwitz et al., 2005, Bartosh et al., 2008). Furthermore, ES cells and CSCs have been found to move toward cardiac lineages when co-cultured with MSCs (Hatzistergos et al., 2010, Yue et al., 2010). In all of these systems there is direct contact between the cell populations. However, this technique would have been technically difficult in downstream analysis and furthermore in primary dog cells direct co-culture may allow for the development of cell fusion which may be a significant limitation of previous studies using this technique. We therefore decided to co-culture indirectly, thereby testing soluble factors released from MSCs and their influence upon CSCs.

Morphologically CSCs showed little difference after 6 days co-culture, compared to controls. However by day 10, there was a more heterogeneous morphology seen in co-cultured wells, with irregular growth patterns with cobblestone appearance when compared to control wells, which showed cells starting to align. This was seen in all wells. In terms of marker expression, there was some variation in specific markers. CD45, a haematopoietic marker appeared to decrease with time in culture, decreasing in the population of cells derived from one dog more quickly than the other. Given this is seen in both co-culture and control wells, this may be either related to time in culture or adherent culture. C-Kit expression was at low levels in day 0 CSCs and disappeared completely by day 6. By day 10 faint expression had returned, and showed variable expression as seen in our previous experiments. The cardiac development markers GATA 4, Nkx2.5 and Flk-1, and the smooth muscle marker glomulin all appeared to increase expression over time in culture, which may suggest movement toward cardiac differentiated lineages. There was however a stronger expression of Nkx2.5 in control wells, and an increased expression of Flk-1 in co-culture wells suggesting that co-culture may delay or decrease cardiomyocyte directed differentiation. Interestingly, vWF showed increased expression in co-culture wells, and when combined with the Flk-1 result may suggest that co-culture with MSCs are driving the CSCs toward a more endothelial cell type, which may also be reflected in the cellular morphology. Furthermore, VEGF splice variant banding appeared similar to CSCs throughout the experiment, however some variation in band strength is seen which may be related toward cell differentiation as previously discussed, and the relevance of which is to be

investigated in future work. No expression of cardiac functional genes was seen. In summary, there is some suggestion of differentiation being triggered using indirect co-culture with MSCs, however this differentiation may in fact be toward cardiac endothelial lineages rather than cardiomyocytes.

4.4.6 Conclusion

We have shown that cells which form cardiospheres can be grown from canine cardiac tissue in culture. Our results indicate that these cells have key characteristics of stem cells both in terms of cell behaviour and marker expression. These cells are capable of maintaining these characteristics in extended culture in serum free media, and of clonal expansion and asymmetric division. Using two alternative differentiation protocols, it has been seen that the CSCs can be driven toward a cardiomyocyte lineage, with downregulation of cardiac stem cell markers, and an increase in cardiac lineage markers using 5'AZA, and may be driven toward endothelial lineages using indirect co-culture with MSCs. However, in both systems the differentiation process did not produce viable terminally differentiated cell lineages, and therefore the technique required to drive these processes will need to be investigated further.

These experiments strongly support the use of canine cardiac stem cells in further studies. The changes exhibited by the cells following the differentiation protocols are very encouraging and we will be undertaking further research to fully elucidate the

conditions required for true differentiation of the stem cells into functioning cardiomyocytes.

In conclusion we have provided compelling evidence that the canine heart has a resident population of adult stem cells, which can be reliably and reproducibly isolated and cultured from atrial explants. This cell population in terms of marker expression has many similarities with other described stem cell populations including the human and thereby may well provide a realistic cell population for *in vitro* veterinary studies and act as a large animal model for cardiac stem cell research.

CHAPTER 5

Canine Mesenchymal Stem Cells and the Cardiovascular System

Abstract

Mesenchymal stem cells (MSCs) have a capacity for multi-lineage differentiation thus providing a promising source of stem cells for regeneration. This chapter initially discusses the isolation of MSCs from different species and sources, and the use of MSCs as alternative to CSCs for cardiac disease investigation and therapeutics given the limitations in cell number and difficulty in obtaining these cardiac cells (discussed in Chapter 4). This chapter then describes the isolation and detailed characterisation of primary canine MSCs. Secondly this chapter investigates the ability of canine MSCs to differentiate toward cardiac lineages. Canine MSCs were isolated from bone marrow aspirates and cultured using traditional techniques. Canine MSCs behaved in culture and appeared morphologically similar to previously described MSCs from other species. Furthermore, canine MSCs demonstrated a marker panel similar to other MSC populations including expression of CD44 and STRO-1, and negative for CD34 and CD45. Canine MSCs were subsequently exposed to a standard cardiac differentiation protocol with no significant alteration in marker profile seen indicating that cardiac

differentiation was not achieved. Furthermore canine MSCs were grown in co-culture with canine CSCs, and again, no evidence of cardiac differentiation was seen using the criteria set out in this study.

This study highlights the ease of obtaining an alternative adult stem cell source from the dog which may be easily isolated and cultured, and closely resembles MSCs described from other species however the differentiation potential of these cells will need to be investigated further.

5.1 Introduction

Mesenchymal stem cells (MSCs) are derived from the mesodermal lineage, and offer an opportunity to study adult stem cells *in vitro*. MSCs have a capacity for multi-lineage differentiation and therefore provide a promising source of stem cells for regenerative medicine. Furthermore MSCs have been shown to have a paracrine effect upon damaged tissues which may be of great therapeutic potential.

5.1.1 Mesenchymal Stem Cells

It was recognized in the 1960's that the bone marrow compartment held cells which were capable of regeneration. Irradiated mice were injected with bone marrow cells and the subsequent colonies that formed in the spleen counted. This work suggested that the cell colonies formed were in proportion to the number of cells injected, and that each colony was derived from a single cell with a multiple differentiation potential (Becker et al., 1963). This work led onto the development of bone marrow transplants for treatment of haematopoietic disease, which was a major breakthrough in the treatment of leukaemia (Mathe et al., 1963). Adult stem cells (ASCs) were subsequently described formally in the 1970s, where multiple cells from within the haematopoietic compartment were found to have been derived from a common precursor – a stem cell (Fialkow, 1973). Bone marrow derived stem cells capable of mesodermal differentiation were classified by Caplan *et al* in 1991 as MSCs, and were capable of differentiation along three main routes; osteoblast, chondrocyte and adipocyte (Caplan, 1991).

5.1.1.1 Mesenchymal Stem Cell Source

Since the original isolation of MSCs from the bone marrow compartment, they have been isolated from other sites within the body. Two major locations are used commonly as an alternative to the bone marrow: Adipose derived MSCs can be obtained in greater number, require less invasive collection techniques (Pittenger et al., 1999, Strem et al., 2005) and are capable of extended time in culture with low levels of senescence allowing maximization of cell numbers from original sampling and extended time experiments (Zuk et al., 2002); Umbilical cord blood MSCs require no invasive surgical procedure but can only be harvested during birth. MSCs collected from both these locations are genotypically and phenotypically similar to bone marrow derived MSCs, and are capable of multipotent differentiation (Kestendjieva et al., 2008, Seo et al., 2009). Other locations have also been described, including circulating MSCs and those isolated from mesodermal compartments such as muscle although less information is available concerning these cell populations (Bosch et al., 2000, Kuznetsov et al., 2001).

5.1.1.2 Defining Mesenchymal Stem Cells

MSCs have traditionally been described using marker expression panels. However the range of markers utilized by different investigators makes the distinction challenging when isolating a single common stem cell population. Furthermore, isolation is traditionally based upon adherence to tissue culture plastic, but in the case of murine

MSCs, these are usually pre-selected against CD11b, therefore making inter-species comparisons difficult (Kopen et al., 1999, Baddoo et al., 2003, Tropel et al., 2004). Due to the increased variation in MSC populations described, the International Society for Cellular Therapy released a document stating the minimum criteria required to define human MSCs, the key features of which were; cells must express a defined marker panel (Table 5.1); cells must be isolated using tissue culture plastic adherence; finally, cells must be capable of differentiation into osteoblasts, chondrocytes and adipocytes (Dominici et al., 2006).

Table 5.1. Minimal marker expression profile for human MSCs (Taken from (Dominici et al., 2006))

Positive	***Negative***
CD105	CD45
CD73	CD34
CD90	CD14 or CD11b
	CD79α or CD19
	HLA-DR

Two further markers commonly used to describe MSCs are CD44 and STRO-1. CD44 is a glycoprotein surface marker, and has been shown to be expressed by MSCs isolated from all three common compartments and from different species (Pittenger et al., 1999,

Meirelles Lda and Nardi, 2003, Kern et al., 2006). STRO-1 has been described as a positive marker used to isolate a cell subset from human bone marrow with MSC properties, allowing for a purer starting cell population (Simmons and Torok-Storb, 1991, Gronthos et al., 1994, Dennis et al., 2002)

5.1.2 Multipotent Differentiation of Mesenchymal Stem Cells

5.1.2.1 Mesodermal

MSCs are capable of differentiation into several mesodermal lineages *in vitro*. In particular connective tissue cell types are readily produced using well described techniques; osteoblast, adipocyte, chondrocyte and myocytes have all been produced from MSCs (Johnstone et al., 1998, Pittenger et al., 1999, Gang et al., 2004). Mesodermal differentiation of MSCs is discussed in more detail in Chapter 7; MSC to Cartilage Differentiation

5.1.2.2 Non-connective Tissue

It has also been suggested that MSCs are capable of differentiation into non-connective tissue cell types. Astrocyte and neuronal differentiation *in vivo* supports the potential of using MSC sources for a multitude of neuronal disorders (Kopen et al., 1999, Jiang et al., 2002, Zeng et al., 2011). Furthermore, MSCs may be capable of differentiation into

hepatocytes representing a future source of cells for treatment of hepatic disorders (Sato et al., 2005).

5.1.2.3 Cardiac Differentiation of Mesenchymal Stem Cells *In Vitro*

Cardiac disease is one of the biggest causes of morbidity and mortality in dogs and humans. As already discussed in Chapter 4, adult stem cells may offer an opportunity to study disease processes *in vitro* and ultimately be used in therapeutics. CSCs are capable of cardiac directed differentiation, however are limited by the difficulty in isolation and the low cell numbers achieved in culture. MSCs may offer a realistic alternative to CSCs for both *in vitro* studies and therapeutics given their ease of isolation and ability for multipotent differentiation.

Using rodent models it has been suggested that MSCs are capable of cardiac differentiation *in vitro* using the de-methylating agent 5'AZA, demonstrating an increase in cardiac specific genes and rhythmical beating (Makino et al., 1999, Fukuda, 2001, Kruglyakov et al., 2006). Furthermore, the addition of cardiotrophin 1 has been found to have an additive effect upon 5'AZA, improving efficiency of cardiac differentiation (Xinyun et al., 2010). This work has been repeated using human MSCs demonstrating successful cardiac directed differentiation with increased expression of cardiac specific genes such as β-MHC and α-cardiac actin and although no spontaneous beating was seen a rhythmical calcium fluctuation was demonstrated (Xu et al., 2004, Antonitsis et al., 2007).

Co-culture with RNCM is a common alternative differentiation technique to using 5'AZA. Co-culture of human MSCs with RNCMs triggered an upregulation of cardiac troponin T and GATA 4, but not cardiac troponin I and myosin heavy chain, which may reflect only partial differentiation (Koninckx et al., 2009). Direct co-culture of rat MSCs with both cardiomyocytes and smooth muscle cells triggered differentiation toward the tissue the cells were co-cultured with. This could not be recapitulated during indirect co-culture, suggesting a requirement for cell-cell contact (Wang et al., 2006). However, in the reverse experiment, MSCs have been found to trigger cardiac differentiation of ES cells, using both direct co-culture and MSC conditioned medium, which suggests that soluble released factors from MSCs do have an influence over differentiation (Yue et al., 2010).

Alternative differentiation methods have been described including nitric oxide agents, hypoxia and conditioned media all with differing degrees of success (Shim et al., 2004, Xie et al., 2006, Rebelatto et al., 2009).

5.1.2.3.1 Defining Differentiation

The criteria used to define differentiation varies through the literature, but is generally based upon an increase in cardiac lineage marker expression, and behaviour and morphology in culture. MSCs have been isolated from different locations, and taken from different species. Therefore making comparisons between cell populations is

reliant upon consistency in marker expression. However, markers used to define differentiation vary and also criteria such as evidence of beating are not always used when stating successful differentiation. The lack of fixed criteria used to define differentiation does make it more challenging to interpret these studies. It must also be borne in mind that in both direct co-culture *in vitro* and direct implantation of MSCs into the heart *in vivo* (particularly in species whereby colour labeling of cell populations in impossible) there is a possibility of cell fusion, thus skewing any marker expression results and beating analysis (Oh et al., 2003, Rose et al., 2008).

5.1.2.3.2 Pathways of Differentiation

Cardiac differentiation of MSCs is a complex process with a multiplicity of pathways playing critical roles. Although cardiac directed differentiation of MSCs has been described using several techniques, little is known regarding the specific pathways involved.

Some information is available from the limited studies performed and can be summarised thus:

- Stromal cell derived factor 1 (SDF-1) is a member of the chemokine CXC subfamily and was found to promote cardiac differentiation of human and mouse bone marrow derived MSCs *in vitro,* suggesting that the CXCR4/PI3K/AKT pathways are significant in cardiac differentiation (Chen et al., 2008).

- MSCs increase the expression of c-Kit in neonatal cardiomyocytes via the secretion of insulin like growth factor 1 (IGF-1) and the PI3K/AKT pathways (Yu et al., 2009b).
- The glycogen-synthase-kinase family has individual and specific effects on cardiac differentiation. GSK3-β has been found to play a critical role in cardiac differentiation of mouse MSCs when found in the cytoplasm through down-regulation of β-catenin of the Wnt pathway, whereas GSK3-α was found to inhibit cardiac differentiation when found in the nucleus by down regulating c-Jun of the JNK pathway (Cho et al., 2009).
- Furthermore, injection of GSK3-β overexpressing MSCs into damaged myocardium improves the efficiency of MSC mediated repair, by increasing survival of MSCs and angiogenesis (Cho et al., 2011).

From this summary it can be seen that there are many different pathways involved in cardiac directed differentiation of MSCs, and that each pathway is crucial but further work is required to examine the interplay between each specific factor involved.

5.1.3 The Use of Mesenchymal Stem Cells in Cardiac Disease

Due to their ease of isolation, the variable locations they can be cultured from and the proposed ability for cardiac differentiation there has been extensive investigation of this ASC source as a therapeutic agent and as a source of cellular precursors. Human bone

marrow stem cells were found to differentiate into cardiomyocytes when transplanted into the adult mouse heart (Toma et al., 2002) and green fluorescent protein (GFP) labeled mouse bone marrow cells injected intravenously into mice with an induced myocardial infarction were found to assist in restoration of cardiac function parameters and cardiac remodelling (Orlic et al., 2001a).

Animal models and clinical trials in humans using MSCs in cardiac disease have suggested a short term improvement in some cases. Injection of MSCs into porcine infarcted hearts differentiated into cardiomyocytes and vascular structures, and also upregulated the presence of c-Kit positive CSCs (Hatzistergos et al., 2010). The current rodent models are poor substitutes for direct comparisons to humans in terms of both size and cardiac physiology and as previously discussed larger animal models are being utilized including the pig and the dog, and data generated from these studies has the potential to be used in veterinary medicine.

5.1.3.1 Canine Mesenchymal Stem Cells and Cardiac Disease

Canine MSC isolation was first described by Kadiyala *et al* in the mid 1990's. These cells were isolated using standard human techniques of tissue culture plastic adherence and were capable of osteochondrogenic differentiation (Kadiyala et al., 1997). Following their isolation, a large amount of work was performed investigating their use for joint and cartilage diseases; but more recently the use of canine MSCs for cardiac diseases has been investigated. Currently, dogs are being used as models for human disease

research, and the results from these studies can be directly translated into veterinary medicine. Bone marrow derived MSCs need to be investigated for their safety when used for cardiac therapy in dogs, and route of delivery, cell type and cell number are all critical factors. Currently conflicting literature surrounds the use of MSCs in canine heart disease, with one study seeing minimal fibrotic change when autologous MSCs were injected directly into myocardium (Li et al., 2003) whereas acute myocardial ischaemia and subacute myocardial microinfarction were seen in an alternative study when autologous MSCs were injected into the heart via the coronary artery (Vulliet et al., 2004).

In a chronic ischemia study using dogs as a model, allogeneic bone marrow derived MSCs were injected into chronically infarcted myocardium and were found to improve vascularisation and cardiac function over 60 days (Silva et al., 2005). Human MSCs transfected with pacemaker genes were transplanted into the heart of dogs with induced heart block (whereby the electrical signal cannot pass through the heart tissue). In these dogs pacemaking function was seen for up to 6 weeks of study, providing a possible alternative to the use of electrical pacemakers (Plotnikov et al., 2007). These results may suggest a positive effect of MSCs in longer term repair of the heart, counteracting the acute damage previously described.

In two further studies utilizing human MSCs injected into the canine heart, it was found that MSCs home to the site of ablation following radio-ablation therapy and differentiate into cardiomyocyte-like cells (Kim et al., 2011) and that MSCs laced onto an

extra-cellular matrix scaffold can be used to repair full-thickness right ventricular defects (Potapova et al., 2008). No data is available on the clinical benefits of MSCs injected into canine hearts for the treatment of naturally occurring congenital or acquired canine cardiac diseases but based on the preliminary data from experimental studies and findings from human clinical trials this is an area worthy of further study.

5.1.3.2 Mobilisation of Native Mesenchymal Stem Cells in Heart Disease

Cells expressing cardiac lineage markers Nkx2.5, GATA 4 and MEF2C reside in the bone marrow of young mice and humans, and decrease in number with age. These cells come from the non-adherent CXCR4 positive, CD45 negative non-haematopoietic mononuclear cells and it is proposed that these cells have the propensity for cardiac regeneration following mobilization after cardiac injury (Kucia et al., 2004). It has been observed that in humans following acute myocardial infarction, an increase in circulating MSCs occurs. This mobilization is heavily influenced by the expression of SDF-1 signaling to the bone marrow to release CXCR4 positive muscle progenitor cells which home to the heart (Wojakowski et al., 2004). Also the KitL ligand (stem cell factor, SCF) is released in response to cardiac injury, which stimulates the activation of c-Kit positive MSCs to be released from the bone marrow and these cells home to the damaged myocardium and release pro-angiogenic factors (Fazel et al., 2008). This data suggests that there is potential for MSCs present in the bone marrow to generate repair in the heart following injury, and optimising this cell population may allow for autologous cardiac therapies without the need for surgical intervention.

5.1.4 The Paracrine Effects of Mesenchymal Stem Cells in the Heart

It is believed that MSCs exert a paracrine effect upon cardiac tissue which modulates and controls inflammation and fibrosis following damage, and as previously mentioned MSCs are thought to translocate from the bone marrow to the heart following damage signals. Furthermore, it is believed MSCs secrete several factors important for cell survival and immunomodulation (Figure 5.1) (Meirelles Lda et al., 2009, Mirotsou et al., 2011).

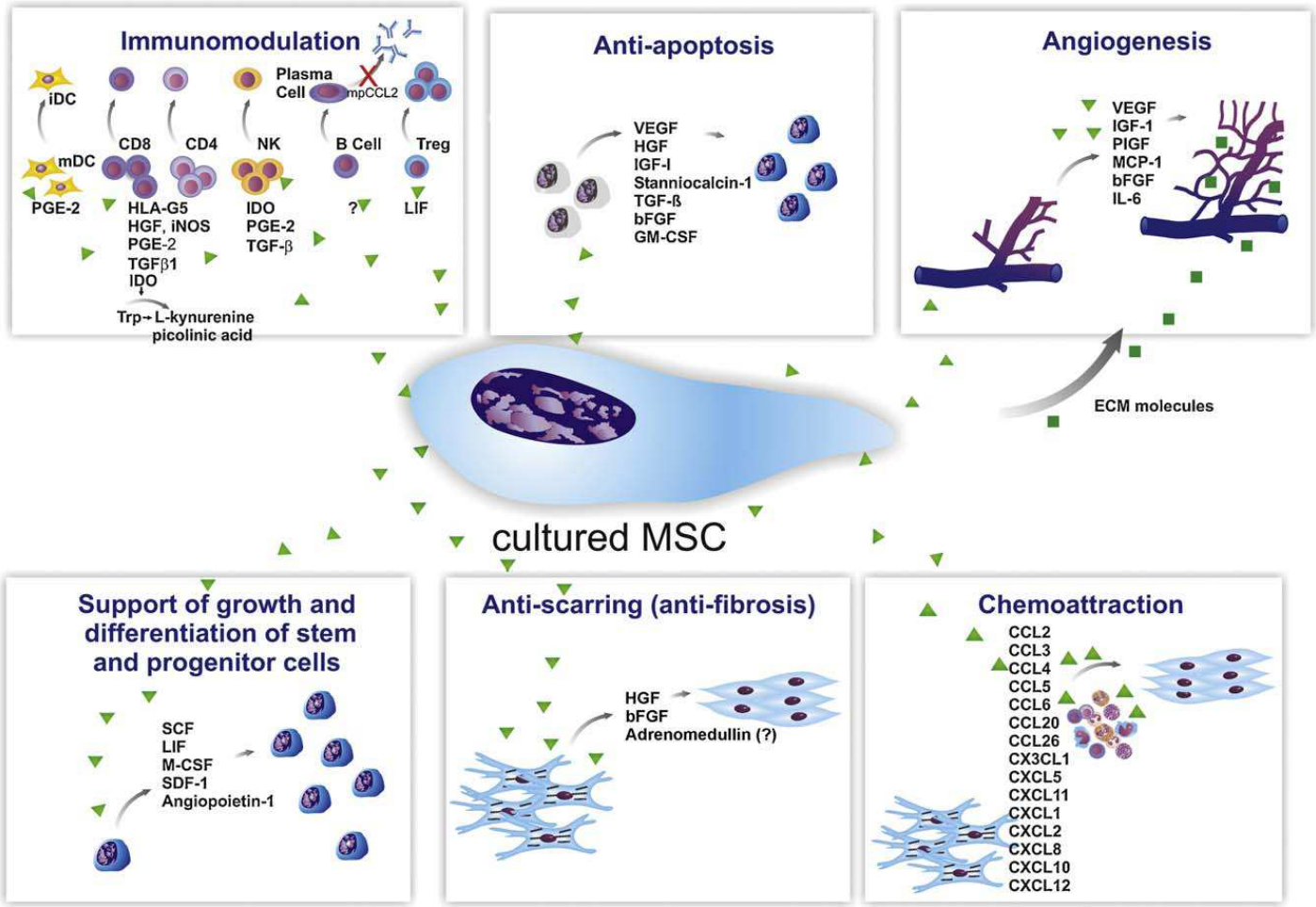

Immunomodulation
iDC
mDC
PGE-2
CD8
CD4
NK
B Cell
Treg
Plasma Cell
mpCCL2
HLA-G5
HGF, iNOS
PGE-2
TGFβ1
IDO
IDO
PGE-2
TGF-β
?
LIF
Trp→ L-kynurenine
picolinic acid
Anti-apoptosis
VEGF
HGF
IGF-I
Stanniocalcin-1
TGF-ß
bFGF
GM-CSF
Angiogenesis
VEGF
IGF-1
PIGF
MCP-1
bFGF
IL-6
ECM molecules
cultured MSC
Support of growth and differentiation of stem and progenitor cells
SCF
LIF
M-CSF
SDF-1
Angiopoietin-1
Anti-scarring (anti-fibrosis)
HGF
bFGF
Adrenomedullin (?)
Chemoattraction
CCL2
CCL3
CCL4
CCL5
CCL6
CCL20
CCL26
CX3CL1
CXCL5
CXCL11
CXCL1
CXCL2
CXCL8
CXCL10
CXCL12

Figure 5.1. Paracrine effects of MSCs in cardiac disease. A host of factors are released from tissue and from the MSCs themselves in response to damage, triggering several events which limit scar formation and promote healing (see abbreviations list for specific factors). Immunomodulation occurs via the inhibition of T lymphocytes, natural killer (NK) cells and dendritic cells (iDC and mDC) and the suppression of immunoglobulin release. Apoptosis is limited and angiogenesis stimulated to maintain and provide blood flow to the damaged areas. Tissue specific stem cells are activated by MSCs and scarring limited by anti-fibrotic mediators. The release of a host of chemokines attracts leukocytes to the damaged tissue to maintain normal tissue viability (Meirelles Lda et al., 2009).

Specifically certain key factors have been shown to be critical for cardiac protection. Matrix metalloproteinases (MMPs) are released during cardiac damage and these were found to activate c-Kit in bone marrow derived MSCs (Fazel et al., 2008). MSCs were also found to stimulate the activity of MMPs and decrease the viability of cardiac fibroblasts, thereby decreasing collagen secretion. MSCs also decreased the expression of MMP inhibitors released from cardiac fibroblasts, reducing the fibrotic damage seen in cardiac disease (Mias et al., 2009). MSCs modified to over-express Akt have been found to reduce infarct size in rodent models following cardiac injury, and it is believed that the Secreted frizzled protein (Sfrp 2) is a key factor in this effect (Gnecchi et al., 2005). Sfrp 2 is an upstream regulator in the canonical Wnt pathway, and cardiomyocytes treated with this protein increase cellular β-catenin and the expression of anti-apoptotic genes (Mirotsou et al., 2007). MSC conditioned media has beneficial

effects on the proliferation and survival of cardiac stem cells located within the heart itself, and it is proposed that secreted factors such as HGF, IGF-1 and VEGF may be responsible for these effects (Nakanishi et al., 2008). Notch pathways are crucial in embryonic development in the heart, and it has been proposed that Notch 1 from bone marrow derived MSCs is important in cardiac repair following ischemic damage by the mediation of neovascularisation (Li et al., 2011). It can be seen from this data that the paracrine effects of MSCs are diverse and involve a large number of different factors and pathways, and much cross-talk between pathways appears to occur. This is a developing field and the future may include the development of released factors as specific targeted therapies, as has been proposed by He *et al.* This group showed that exogenously administered Sfrp 2 significantly reduced fibrosis following cardiac damage in rodent models; the idea for which was based upon previous studies of MSC over expression of this protein (He et al., 2011).

5.1.5 Mesenchymal Stem Cell Clinical Trials for Cardiac Disease

Clinical trials using MSCs for cardiac disease are already underway and overall demonstrate a trend for generalized short term improvement. However these trials have been undertaken following limited basic stem cell science research making it difficult to ascertain at this stage whether the improvements seen are caused by a paracrine effect promoting scar remodeling versus a replacement/renewal effect, or perhaps a combination of both. Trials include the REGENERATE, TOPCARE, ASTAMI and BOOST trials (Schachinger et al., 2004, Meyer et al., 2006, Assmus et al., 2007, Lunde

et al., 2007, Yeo and Mathur, 2009). These trials all suggest a generalised improvement in measurable outcomes in patients with cardiac disease, such as increased ejection fraction and decreased end-systolic volume.

A separate trial has found that injection of bone marrow derived stem cells into the coronary artery following an acute myocardial infarction does not improve measurable outcomes as before, however they do promote infarct remodeling and the significance of which will need to be investigated in follow up of this patient group (Janssens et al., 2006).

Long term follow up of these MSC treated patients is still ongoing, and results of these studies will be critical in deciding the future of MSC therapy in cardiac disease. Further work elucidating direct and indirect effects of MSCs within the damaged heart must be performed.

5.1.6 Aims

This chapter describes the isolation and culture of canine primary MSCs from the bone marrow. Detailed characterisation of the isolated population will be performed and the differentiation potential toward cardiac lineages investigated.

5.2 Materials and Methods

5.2.1 Isolation and Culture of Canine Mesenchymal Stem Cells

5.2.1.1 Tissue Samples

Femurs from recently euthanized dogs were dissected and exposed from the lateral thigh, and the shaft split using a small hacksaw distal to the femoral head and proximal to the femoro-patellar joint, releasing a mid-diaphyseal section. The medullary cavity of these sections were washed with standard mesenchymal stem cell media; DMEM low glucose containing Glutamax-I (Invitrogen, UK), with 10% FBS (Invitrogen, UK) and 100 U/mL penicillin G and 100 μg/mL streptomycin (both Invitrogen, UK), and 17 U/mL of Heparin (Wockhardt Ltd, UK), and chilled on ice. If possible cells from femoral sections from both hind legs were pooled to maximize cell number.

5.2.1.2 Sample Preparation

Heparinised media containing bone marrow cells was made up to 10 mL total volume using pre-warmed PBS, and layered onto 16 mL of Ficoll Paque Premium (GE Life Sciences) in a 50 mL Falcon tube. The samples were centrifuged without brake setting for 30 minutes at 450g. The interface layer was collected using Pasteur pipettes and transferred to a 15 mL Falcon tube and made up to 8 mL using PBS. This was then centrifuged for 5 minutes at 150g to pellet the cells. The PBS was removed and

discarded and the cell pellet re-suspended in 1 mL of warmed MSC media, and the cells counted as previously described using white cell counting mount solution (1% w/v crystal violet and 2% v/v glacial acetic acid in distilled water).

5.2.1.3 Isolation of Stem Cells

Cells were plated at approximately 1 x 10^4 cells/T25 and placed at 37 °C, 5 % CO_2. Cells were left for 2-4 days to allow the adherent cell layer to attach, and then media containing dead cells and debris was removed and discarded and the adherent cells washed using PBS. Fresh MSC media was replaced, and the cells washed every 2-3 days until cells were 80-90% confluent (usually 1-2 weeks). Cells were then passaged using 0.05% trypsin-EDTA (Gibco, UK) to loosen cells, and counted with a haemocytometer and trypan blue live cell stain (Sigma, UK). Approximately 1 x 10^3 cells/cm^2 was replaced at each passage in tissue culture flasks. An increase of approximately 1 log scale of cells was achieved between passage, and 4-5 passages were possible before senescence.

5.2.1.4 Mesenchymal Stem Cell -80 ℃ Storage

Early passage (<3) canine MSCs were trypsinised from tissue culture flasks as previously described, and counted. Cells were centrifuged for 5 minutes at 1500 rpm to pellet and re-suspended in freezing media; 60% FBS, 30% standard MSC media, 10% dimethyl sulfoxide (DMSO) (Invitrogen, UK). 1 x 10^6 minimum total cells were placed

into 1 mL of freezing media in a cryovial (BioSigma, Italy) and the cells placed at -80 °C in an isopropanol chamber for 24 hours before being transferred into longer term liquid nitrogen storage.

Cells were revived by removing cryovials from liquid nitrogen storage and swirling the vial in a water bath at 37 °C for a maximum of 2 minutes to defrost the freezing media. Pre-warmed standard MSC medium was then added to the vial in a dropwise fashion in a 1:1 ratio and the cell suspension then transferred to a 15 mL Falcon tube. A further 3 mL of MSC medium was added, and the cells centrifuged at 1500 rpm for 5 minutes. The cell pellet was re-suspended in 1 mL of MSC media and the cells counted. Cells were re-plated at approximately 3-5 x 10^6 total cells in a T75 flask. Cells were placed at 37 °C, 5% CO_2, with media refreshes every 3 days until cells were 80-90% confluent and ready for passage.

5.2.2 Mesenchymal Stem Cell Characterisation

5.2.2.1 Transcriptional Analysis of Mesenchymal Stem Cells

MSC RNA was extracted and reverse transcribed, and PCR reactions performed as previously described in Chapter 2; Materials and Methods. MSCs were analysed for expression of several key markers; CD44, CD34, CD45 and c-Kit, and compared to RNA from splenic tissue known to express haematopoietic markers, and therefore acting as a negative control for MSCs.

Furthermore analysis of day 0 MSCs was performed during cardiac differentiation analysis, and included cardiac markers from cardiomyocyte, endothelial and smooth muscle lineages (Table 5.2).

Table 5.2. Canine oligonucleotides used for MSC analysis. CTT-Cardiac Troponin T; Beta 1 Ad-β_1-adrenergic Receptor; Cardiac RyR-Cardiac Ryanodine Receptor; CTI-Cardiac Troponin I; VEGF-Vascular Endothelial Growth Factor; vWF-von Willebrands Factor; SMA-Smooth Muscle Actin. F: Forward Primer. R: Reverse Primer

Marker	Sequence 5'-3'	Size (BP)	Tm (°C)
CD44	F: TAC GCA GGT GTG TGT TCC CAT GT	214	58
	R: GGT TTG CAG CAC AAA TAG CA		
CD34	F: TGA CAC CCC AAG TAC CAT CA	162	60
	R: GGC TCC TTC TCA CAC AGC AC		
CD45	F: AGC TAA GGC GAC AGA GAT GC	157	60
	R: ACT GGG CGG GTC TCT TTT CT		
C-Kit	F: ATA TCC CAA ACC GGA GCA C	193	58
	R: TCA CCG AAG AAT TGA CAT CG		
Islet 1	F: GGT TTC TCC GGA TTT GGA AT	183	57
	R: CAC GAA GTC GTT CTT GCT GA		
Nkx2.5	F: CCA AGG ACC CTC GAG CTG A	185	62
	R: CGA CAG ATA CCG CTG CTG CT		
GATA 4	F: CAA GAT GAA TGG CAT CAA CC	216	57
	R: GGT TTG AAT CCC CTC TTT CC		

Marker	Sequence 5'-3'	Size (BP)	Tm (°C)
Flk-1	F: CCA CCC AGA CTC AGC ATA CA	188	58
	R: CAC TTT TGG AAT CGT GAG CA		
CTT	F: GAA GGA CCT GAA CGA ACT	210	60
	R: CCT CCT GTT CTC CTC CTC CT		
Beta 1 Ad	F: GAG TAC GGC TCC TTC CTG TG	274	61
	R: AGC ACT TGG GGT CGT TGT AG		
Cardiac RyR	F: AAG CGA AGC AGC CCA AGG GT	888	62
	R: TCA GCA AAG TGT GCC GCG CT		
CTI	F: TCT GCG CAT GGC GGA TGA GA	624	62
	R: TTG CGG CCC TCC ATT CCA CT		
VEGF	F: TTC CTG CAG CAT AGC AAA TG	293 (239,311)	57
	R: AGG GAG GCT CCT TCT TCC AG		
vWF	F: CTG GGA GAA GAG AGT CAC GG	235	61
	R: GTG GAT GGA GTA CAC GGC TT		
SMA	F: GGG GAT GGG ACA AAA GGA CA	525	61
	R: GCC ACG TAG CAG AGC TTC TCC TTG A		
Glomulin	F: TTG AAG AGC CCT CTG GAA AA	131	57
	R: ACT CCA AAG GGT GGA CAA TG		
Connexin 43	F: ATG AGC AGT CTG CCT TTC GT	249	58
	R: TCT GCT TCA AGT GCA TGT CC		
GAPDH	F: CAT CAA CGG GAA GTC CAT CT	428	Various
	R: GTG GAA GCA GGG ATG ATG TT		

5.2.2.2 Translational Analysis of Mesenchymal Stem Cells

Canine MSCs (passage 2) were seeded onto 4 well chambered slides (Corning, UK) at a density of 2 x 10^4 cells/well. Cells were cultured for 48 hours at which point cells were approximately 60% confluent. The media was removed and slides were air dried and then fixed directly in 4% paraformaldehyde for 20 minutes, followed by two PBS washes and subsequently placed in 100% ethanol for 5 minutes, followed by 2 x PBS washes to permeabilise the cells. Blocking buffer (10% normal goat serum (Invitrogen, UK); 0.1% Tween 20 (Sigma, UK)) in 1x PBS was applied for 1 hour at room temperature. Primary antibodies; rat anti-dog CD44 (AbD Serotec MCA1042G), and mouse anti-human STRO-1 (R&D Systems MAB1038) diluted at 1:100 and 1:50 respectively in PBST (0.1 % normal goat serum, 0.1% Tween 20 in PBS) were added directly to the slides and incubated at 4 °C overnight in a humidified chamber. Following this slides were washed in PBS twice before secondary antibody was added. Alexafluor 488 goat anti-rat IgG and 568 goat anti-mouse IgM (Invitrogen A11006 and A21043 respectively) were diluted 1:500 in PBS and incubated for 1 hour at room temperature. Nuclear counterstaining was performed using DAPI mount (Vectastain, USA) and slides covered with coverslips (VWR International, USA), and sealed using clear nail varnish. Negative controls were performed using secondary antibody only, with all other procedures performed as for positive samples. Images were taken using the Zeiss Axiovert 40CPL microscope and processed using the Axiovision programme.

5.2.3 Cardiac Directed Differentiation of Mesenchymal Stem Cells using Published Techniques

5.2.3.1 Culture and Harvest for Transcriptional Analysis

Canine MSCs (passage 3) were trypsinised from standard culture and counted as previously described. 5 x 10^4 cells in 0.5 mL of MSC media was added to 1 well of a 24-well plate. Plates were labelled for three time points, 7, 14 and 21 days. Each experiment was run in triplicate. A triplicate set of wells was plated to allow for time 0 control (Figure 5.2).

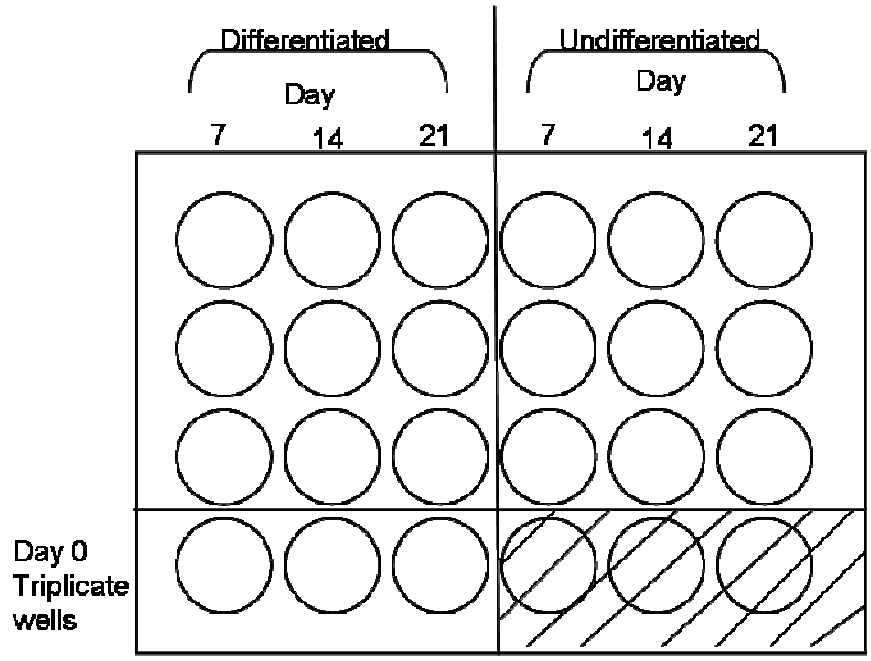


Figure 5.2. Plate plan design for MSC differentiation toward cardiac lineages. Triplicate wells were prepared for day 0, and three further time points, day 7, 14 and 21.

The cells were left overnight in MSC media to allow them to adhere at 37 °C, 5% CO_2. The following day media was aspirated and 2 x PBS washes performed to all wells. Time 0 triplicate wells were trypsinised off, and centrifuged at 1500 rpm, for 5 minutes to pellet cells. Supernatant was removed and discarded, and the cells washed in PBS. The cells were then re-centrifuged to pellet and PBS removed and discarded and the cells snap frozen in dry ice, and stored at -80 °C. 500 μL of MSC media was added to the remaining control wells. 500 μL of MSC media with 3 μmol/L 5'AZA (Sigma, UK) was added to the differentiated wells. Half media changes were performed to all wells, with either standard MSC media or media containing 3 μmol/L 5'AZA every 3 days for 3 weeks (Figure 5.3). Harvests of triplicate wells were taken at days 7, 14 and 21 and pelleted as described for day 0. RNA was subsequently extracted, and triplicate RNA samples pooled for semi-quantitative RT-PCR, performed as described in Chapter 2; Materials and Methods with primers described in Table 5.2. Quantitative RT-PCR was also performed and is described below. Both semi-quantitative and quantitative RT-PCR used 100 ng total RNA.

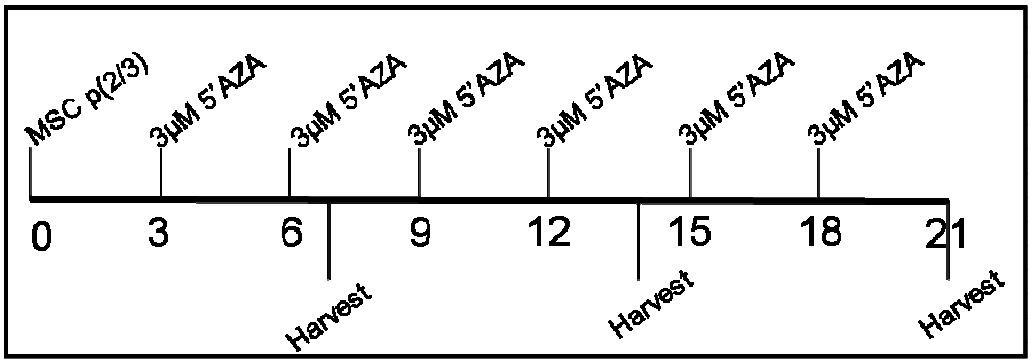


Figure 5.3. Experimental design for MSC differentiation toward cardiac lineage. Following initial set up 3µmol/L 5'AZA was added to differentiating wells every 3 days for 21 days. Harvests were collected weekly, and negative controls were run alongside with no addition of 5'AZA.

5.2.3.2 Quantitative RT-PCR Analysis of Mesenchymal Stem Cell Differentiation

Primers and probes were designed and prepared for Flk-1 and CTT as described in Chapter 2; Materials and Methods, and primers ordered from MWG Eurofins and probes from Roche. Nkx2.5 primer and probe was designed using the Applied Biosystems software, and ordered pre-bound to probe at a 20 X concentration from Applied Biosystems. Sequence details can be found in Chapter 4; Cardiac Stem Cells.

5.2.3.2.1 cDNA Synthesis

Extracted RNA from cardiac differentiation pellets from each time point in triplicate were reverse transcribed as described in Chapter 2; Materials and Methods.

5.2.3.2.2 PCR Master mix

A master mix was prepared for CTT, Flk-1, Nkx2.5 and 18s rRNA controls as described in Tables 5.3, 5.4 and 5.5:

Table 5.3. Target gene master mix for CTT and Flk-1

Master mix	***1x (µL) (n x =number of wells)***
Realtime Master mix	5
10x Primer/Probe (FAM)	0.3

Table 5.4. Target gene master mix for Nkx 2.5

Master mix	***1x (µL) (n x =number of wells)***
Realtime Master mix	5
20x Primer/Probe (FAM)	0.15
Water	0.15

Table 5.5. Target gene master mix for 18s rRNA control gene

Master mix	*1x (µL) (n x =number of wells)*
Realtime Master mix	5
20x 18s VIC Control	0.15
Water	0.15

5.2.3.2.3 Primer Efficiency and Optimisation

Primer pairs were optimized as described in Chapter 4; Cardiac Stem Cells.

5.2.3.2.4 Relative Gene Expression Analysis

Following primer optimization samples could be compared for relative expression of specific gene targets. 100 ng of total RNA of each sample from dog A and B were reverse transcribed and the subsequent triplicate cDNA analysed. A plate plan for relative expression is shown in Figure 5.4.

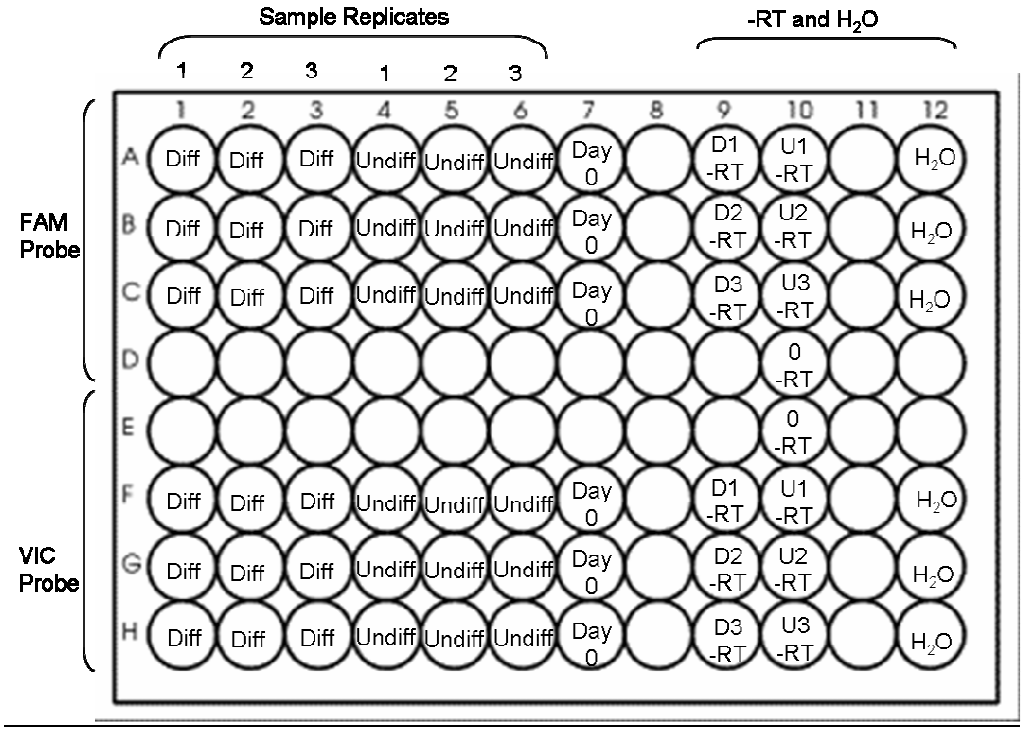


Figure 5.4. 96-well plate plan for gene expression analysis. Wells were designed to allow for triplicates of sample cDNA, differentiated (Diff) and undifferentiated (Undiff) run again in triplicate for technical replicates. Triplicate wells of day 0 cDNA were run alongside as a reference gene. Primers for CTT, Flk-1 and Nkx2.5 were bound to a FAM probe, and primers for 18s rRNA in-house gene positive control bound to a VIC probe. Negative control of water and RNA (-RT) were run on the same plate. One plate was run per dog per time point (day 7, 14 and 21).

This plate design allows for comparison between differentiated (Diff) and undifferentiated (Undiff) samples in two dogs, A and B, over three time points. Day 0 cDNA has been added to allow for a reference control sample. All negatives were run on

the same plate using the same master mix that was used for the samples. All plates used the same cDNA to allow for control across individual plate runs.

Samples were run using programme and cycles described in Tables 5.6 and 5.7;

Table 5.6. Programme data for Roche Lightcycler 480

Programme	*Cycle*	*Acquisition*
Pre-incubation	1	N
Amplification	50	Quantify
Cooling	1	N

With individual cycles as follows:

Table 5.7. Temperature settings per cycle for Roche Lightcycler 480

	Temperature °C	*Acquisition*	*Hold (Time)*	*Ramp*
Pre-Incubation	95	N	10 minutes	4.4
Amplification (50x)	95	N	10 seconds	4.4
	54	N	30 seconds	2.2
	72	Single	1 second	4.4
Cooling	40	N	10 seconds	1.5

5.2.4 Mesenchymal Stem Cell Co-Culture with Cardiac Stem Cells

Canine MSCs were grown in indirect co-culture with autologous CSCs using a transwell system.

5.2.4.1 Harvest and Transcriptional Analysis of Mesenchymal Stem Cells Following Co-Culture

MSCs and CSCs were harvested as described in Chapter 2; Materials and Methods and placed in DEM media and counted.

Following the plate plan as described in Chapter 2; Materials and Methods, 2 x 10^4 MSCs were seeded into base wells of the 24 well plate (growth area 1.9cm^2) in 600 μL of DEM media, with 1 x 10^4 CSCs seeded onto transwell inserts (growth area 0.33 cm^2) in 100 μL DEM media. Control wells of MSCs alone were cultured alongside. Plates were then placed at 37 °C, 5% CO_2. Day 0 MSCs were pelleted at this point, and snap frozen and stored at -80 °C. Images and harvests from both co-cultured and control wells were taken at days 6 and 10 post plating and cells pelleted and snap frozen and stored at - 80°C. RNA was subsequently extracted from pellets and 150 ng total RNA used for semi-quantitative RT-PCR analysis as described in Chapter 2; Materials and Methods for all markers in Table 5.2.

5.3 Results

5.3.1 *In Vitro* Culture of Primary Canine Mesenchymal Stem Cells

5.3.1.1 Morphology and Culture Characteristics

Canine MSCs were isolated from 23 dogs of variable age, breed and sex. Dogs were euthanized for clinical reasons and tissue donated with owners' consent. Following bone marrow harvest into heparinised media, cells were isolated using standardised Ficoll technique and tissue culture plastic plating. MSCs adhered naturally to tissue culture plastic over 2-4 days. Non-adherent (non-mesenchymal) cells floated free and were readily discarded. Morphologically the cells appeared flattened and adherent, with multiple jagged projections (Figure 5.5). These cells were capable of rapid expansion, with log increases in cell number at passage once fully established. Canine MSCs typically senesced between passage 4 and 6, at which point the cells increased in size and the spindle projections would fatten.

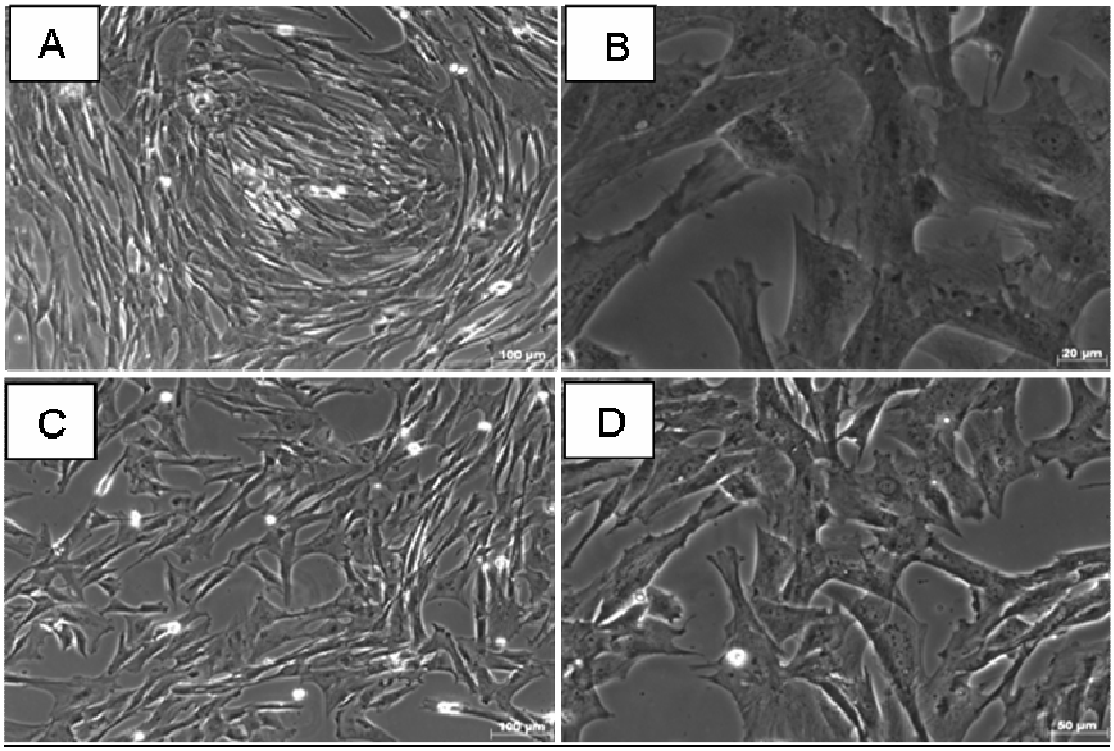


Figure 5.5. Morphology of primary canine MSCs. Canine MSCs resembled previously described MSCs from other species, adhering readily to tissue culture plastic, and forming long, flattened cells with multiple jagged projections. A and C magnification x 100, B magnification x 400, D magnification x 200.

5.3.1.2 Canine Mesenchymal Stem Cell Characterisation using RT-PCR

Canine MSCs were analysed for the expression of the MSC markers CD44, c-Kit, CD34 and CD45 using semi-quantitative RT-PCR and haematopoietic splenic tissue RNA as a control. MSCs were found to be positive for CD44, and demonstrated low expression of c-Kit, CD34 and CD45. Splenic tissue was found to be positive for CD34, CD45, CD44 and c-Kit (Figure 5.6).

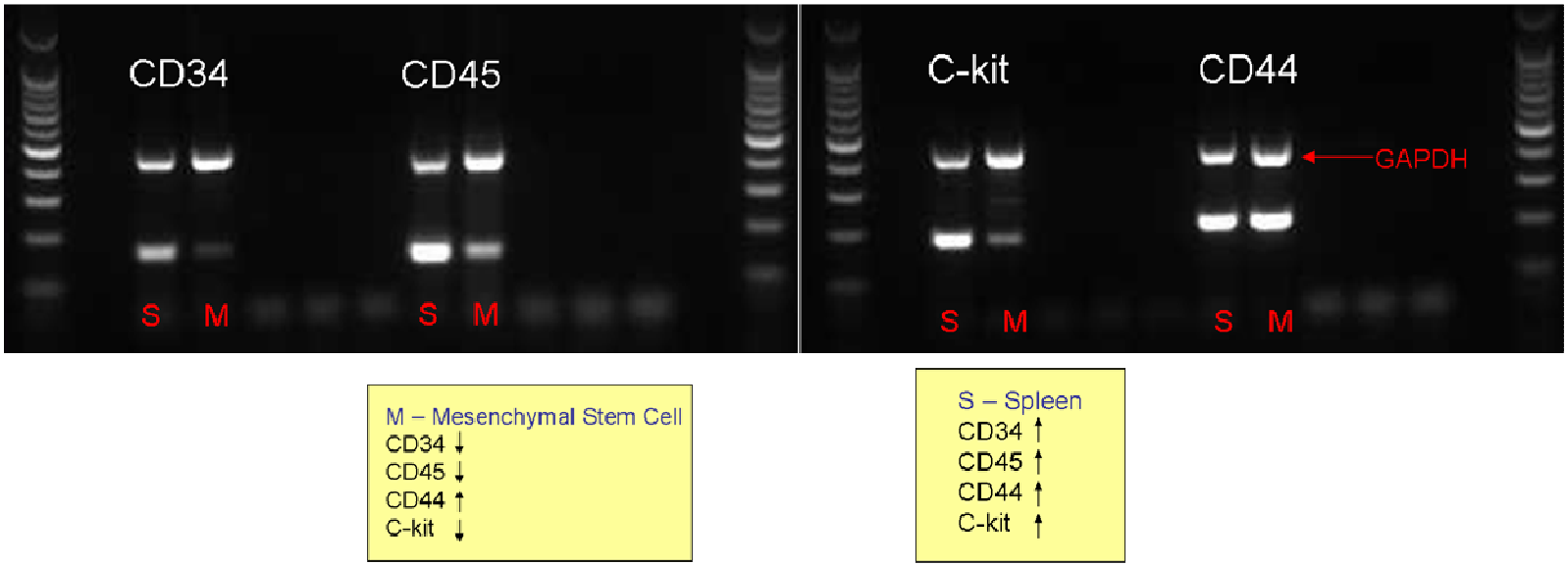


Figure 5.6. Semi-quantitative RT-PCR analysis of MSC (M) expression compared to haematopoietic splenic tissue (S) expression of CD34, CD45, c-Kit and CD44. Each PCR was multiplexed with GAPDH (labelled at 428 base pairs), and bands of appropriate sizes seen for each marker. Expected expression is shown below the PCR image.

5.3.1.3 Mesenchymal Stem Cell Characterisation using Immunofluorescence

Canine MSCs were grown on 4 well chambered slides and analysed for expression of CD44 and STRO-1 at translational level using IFA. Both markers were found to be expressed in the adherent cell population with expression seen at cytoplasmic/cell surface locations (Figure 5.7). Negative controls confirmed absence of non-specific binding

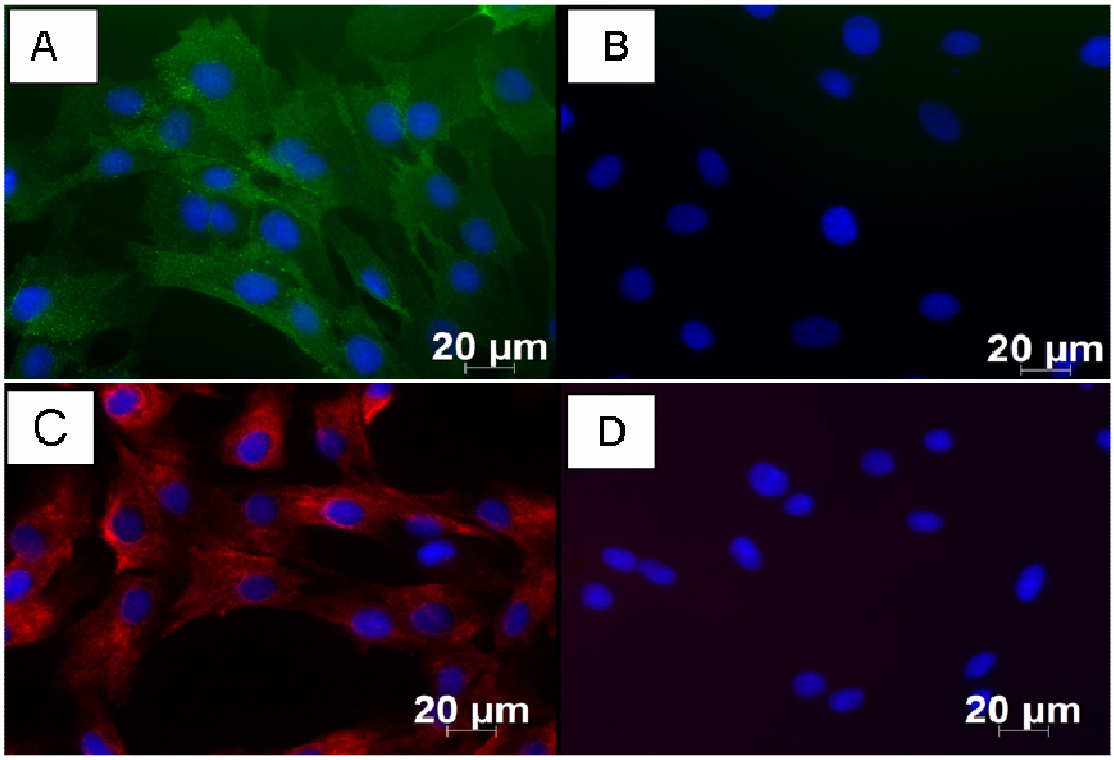


Figure 5.7. Canine MSCs labeled for CD44 and STRO-1. Strong positive staining was seen for both markers. CD44 (A) was seen throughout the surface of the cell. STRO-1 (C) appeared to be found within the cytoplasm of the cells, and particularly dense around nuclei. Negative controls (B and D) for each were performed with secondary antibody only. Nuclei were labelled with DAPI. Magnification x 400.

5.3.2 Cardiac Differentiation of Canine Mesenchymal Stem Cells using Published Protocols

Cardiac differentiation was attempted using the de-methylating agent 5'AZA over a three week time course, based upon published literature.

5.3.2.1 Morphology during Differentiation

Morphologically at early time points (day 7) the cells closely resembled day 0 MSCs in both the test and control populations. By day 14 there appeared to be some suggestion of alignment of the cells in test wells compared to control. By day 21 the lining up appeared more pronounced, with cells swirling in test wells, whereas in control wells cells appeared more irregular however these changes are subjective (Figure 5.8).

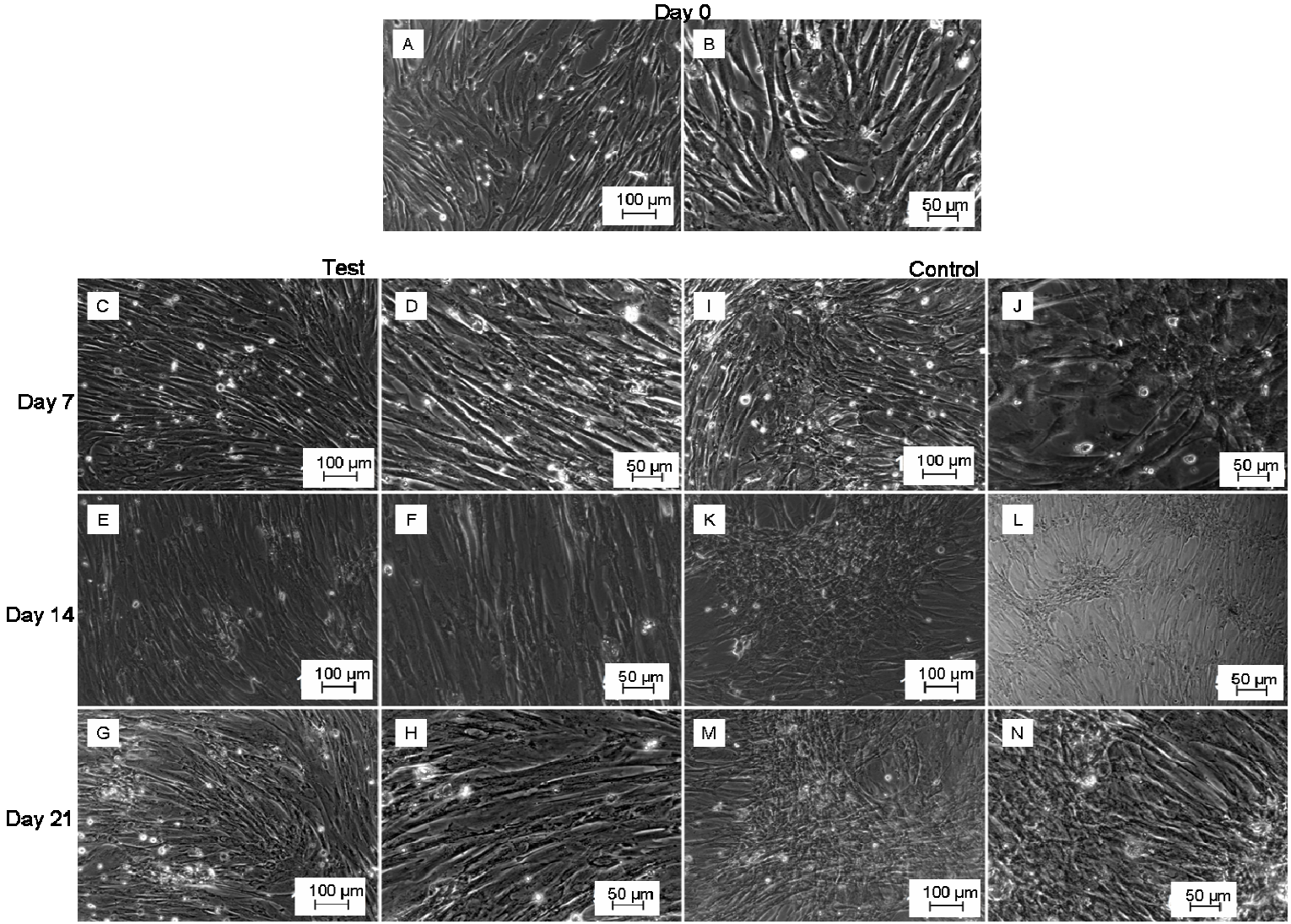

Day 0
A
B
100 µm
50 µm
Test
Control
Day 7
Day 14
Day 21
C
D
I
J
E
F
K
L
G
H
M
N
100 µm
50 µm
100 µm
50 µm
100 µm
50 µm
100 µm
50 µm

Figure 5.8. Cellular morphology during cardiomyocyte differentiation of canine MSCs. Canine MSCs showed minor morphological changes during the differentiation protocol. Some suggestion of cellular alignment was seen by day 14 in the cells undergoing differentiation (Test) (C-H) which was not seen in day 0 (A and B) and control cells (I-O). (Magnification A, C, E, G, I, L and N is x 100; B, D, F, H, J, M, and O is x 200).

5.3.2.2 RT-PCR Analysis of Cardiac Directed Differentiation

Using semi-quantitative RT-PCR, marker expression was examined in two dogs, A and B, following cardiac directed differentiation (Figure 5.9 and Figure 5.10 respectively). In dog A, Nkx2.5 was expressed in day 0 MSCs, which appeared increased in test cells of all time points, with decreased expression in control cells compared to day 0. In dog B there appeared to be lower expression of Nkx2.5 at day 0, which then increased in all cells at all time points. Flk-1 expression appeared low at day 0 in both dogs, and then increased in dog A test cells at days 7 and 21 compared to control. In dog B levels appeared to remain low, with a slight increase suggested in control cells at day 7, and 14. VEGF expression remained constant throughout the differentiation in all cells compared to day 0, and demonstrated multiple banding corresponding to the splice variants of VEGF. SMA, Glomulin, CD44 and Connexin 43 expression levels remained unchanged in test and control samples in both dogs over time compared to day 0 MSCs. In both dogs no expression of GATA 4, islet 1, c-Kit, CD34, CD45, vWF, CTI, CTT, RyR and β_1-adrenergic receptor was seen (data not shown).

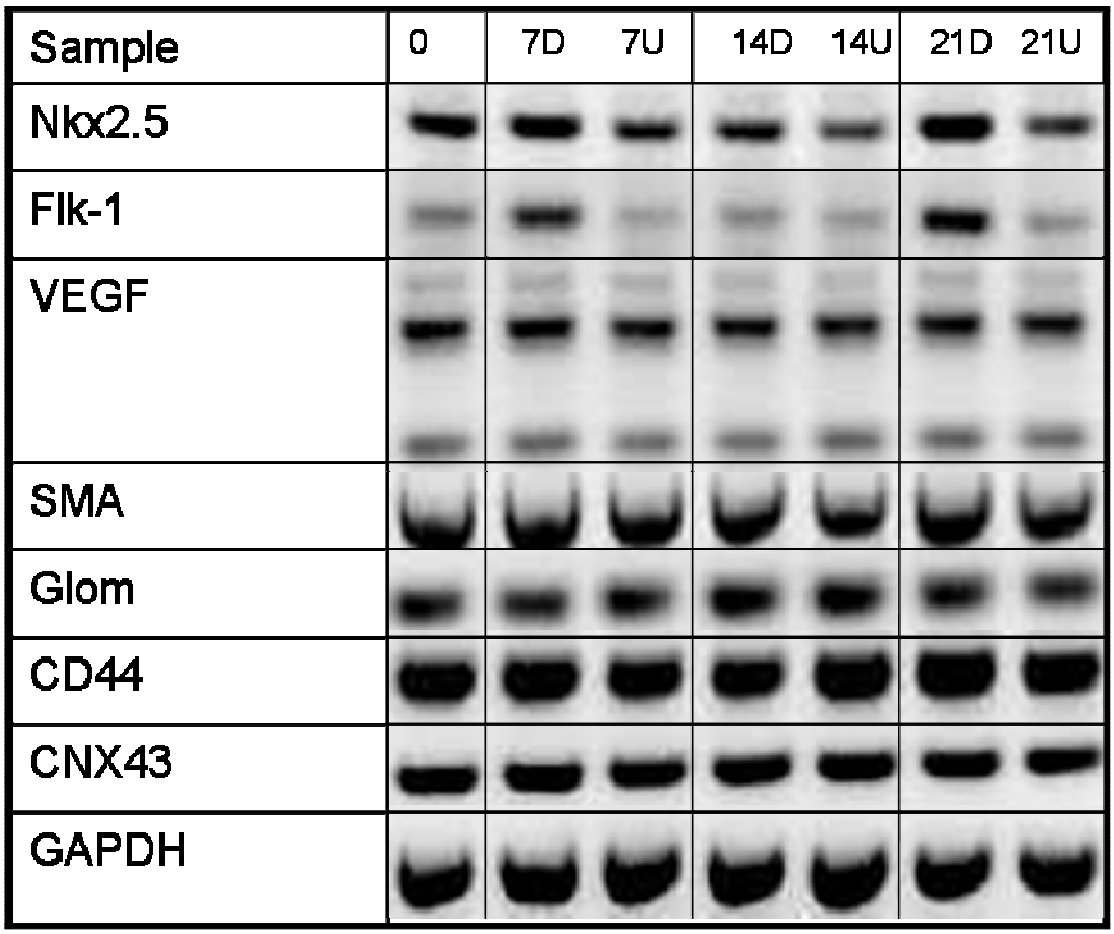


Figure 5.9. Marker expression changes over time during cardiac directed differentiation for dog A. Day 0 (0) baseline MSCs were compared to MSCs undergoing differentiation (D) and those under control conditions (U) at three time points, days 7, 14 and 21. GAPDH was run as an internal multiplexed control.

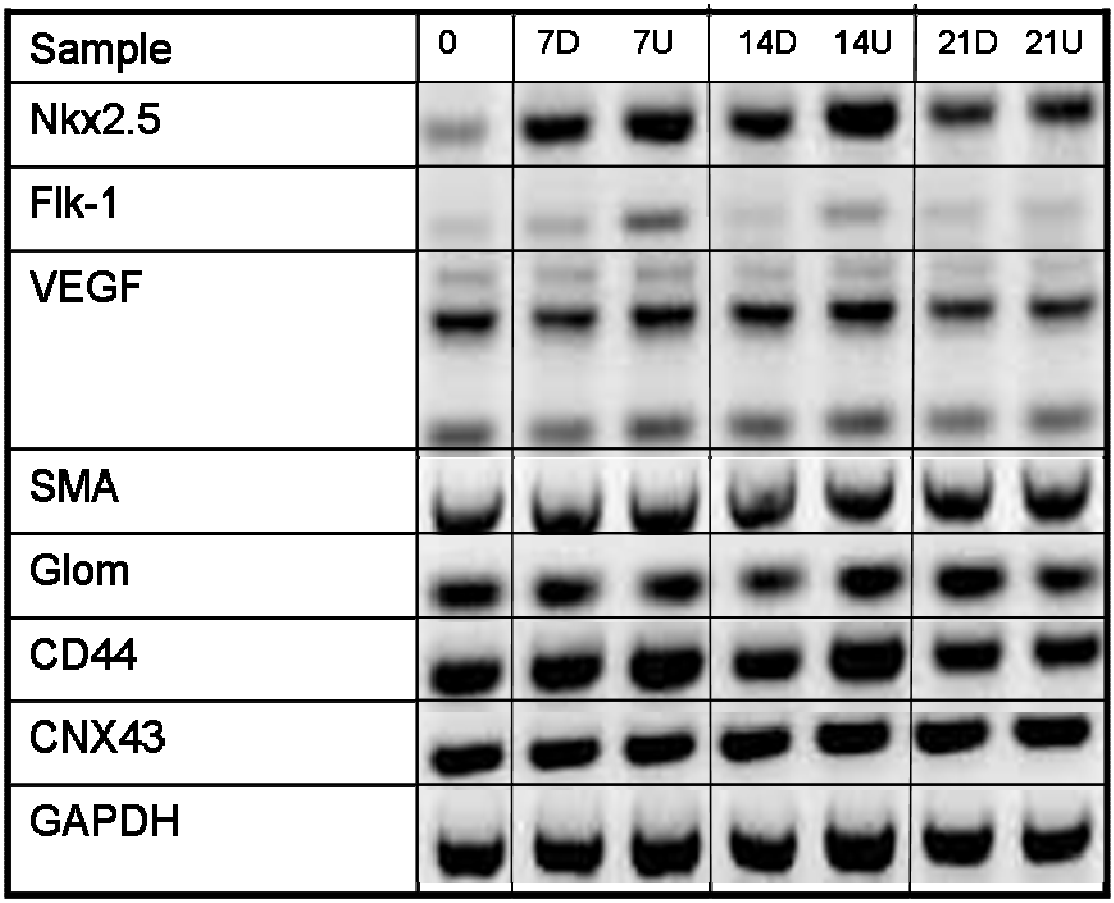


Figure 5.10. Marker expression changes over time during cardiac directed differentiation for dog B. Day 0 (0) baseline MSCs were compared to MSCs undergoing differentiation (D) and those under control conditions (U) at three time points, days 7, 14 and 21. GAPDH was run as an internal multiplexed control.

5.3.2.3 Quantitative RT-PCR Analysis of Cardiac Directed Differentiation

Nkx2.5, Flk-1 and CTT were further examined using quantitative RT-PCR for both dogs A and B (Figure 5.11 and 5.12 respectively). Nkx2.5 generally increased relative to day

0 in cells undergoing differentiation of both dogs compared to controls. Specifically, in dog A at day 14 and 21, there was an approximately 2.5-3 fold increase in test cells compared to day 0, however at day 7 both test and control cells demonstrated this increase. In dog B there was a larger relative increase in both test and control samples, with a fold increase of approximately 6-8 times in test and 5-6 times in control samples at all time points compared to day 0.

Flk-1 expression showed more variability than Nkx2.5, but again showed a general trend for higher expression in test samples than control. Dog A had a 2-3 fold increase in expression in test cells relative to day 0 at days 14 and 21, however at day 7 increase was only 1.5 fold, and was comparable to that of control samples. At days 14 and 21 control samples showed a relative expression of 1 to 1.5 fold, indicating levels similar to that of day 0. In dog B there was a 4 fold increase in test cell expression of Flk-1 by day 7, which dropped to 1.5-2 fold for days 14 and 21. In control cells there was an initial increase to 2-2.5 fold by day 7, which returned to levels comparable with day 0 MSCs at days 14 and 21. There was more fluctuation seen in Flk-1 expression of dog B, with a less pronounced increased expression in test cells compared to controls.

No expression was seen for CTT using quantitative RT-PCR (data not shown) in any of the samples.

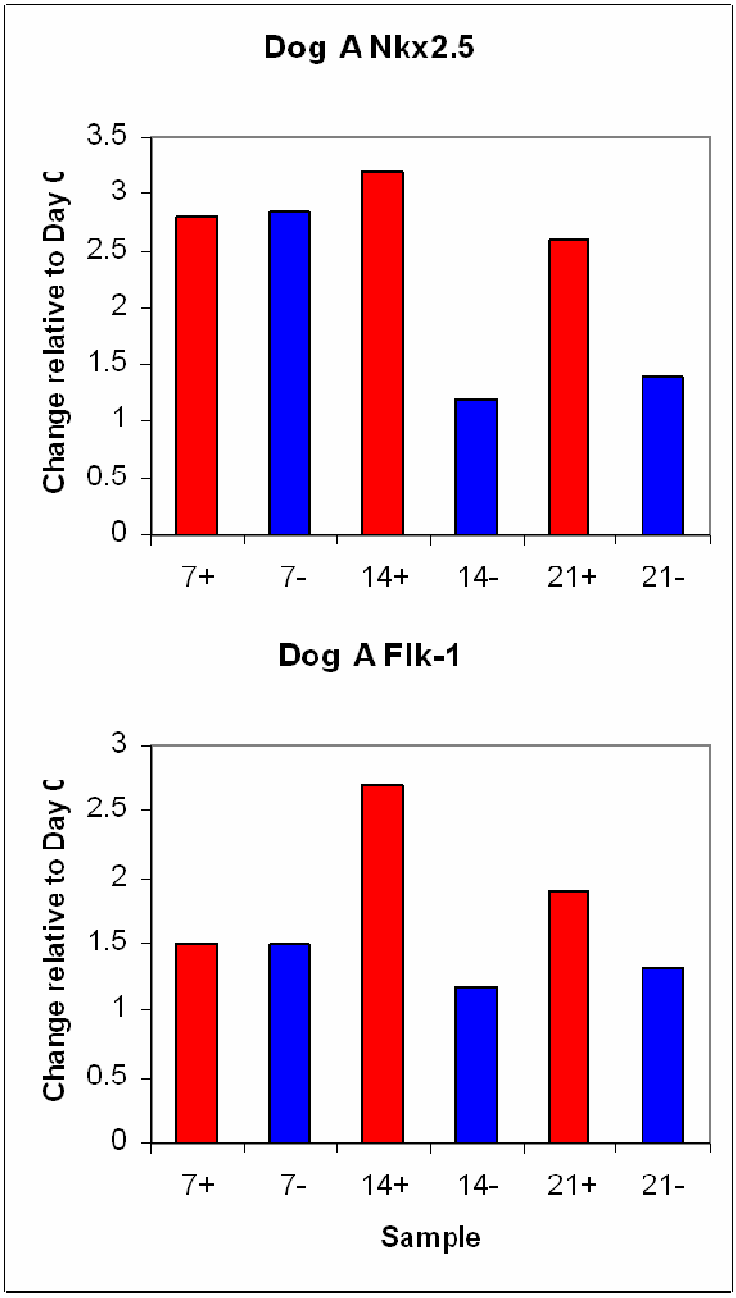


Figure 5.11. Quantitative RT-PCR analysis of dog A expression of Nkx2.5 and Flk-1 during cardiac directed differentiation relative to day 0. Test (+) and control (-) results are shown for days 7, 14 and 21

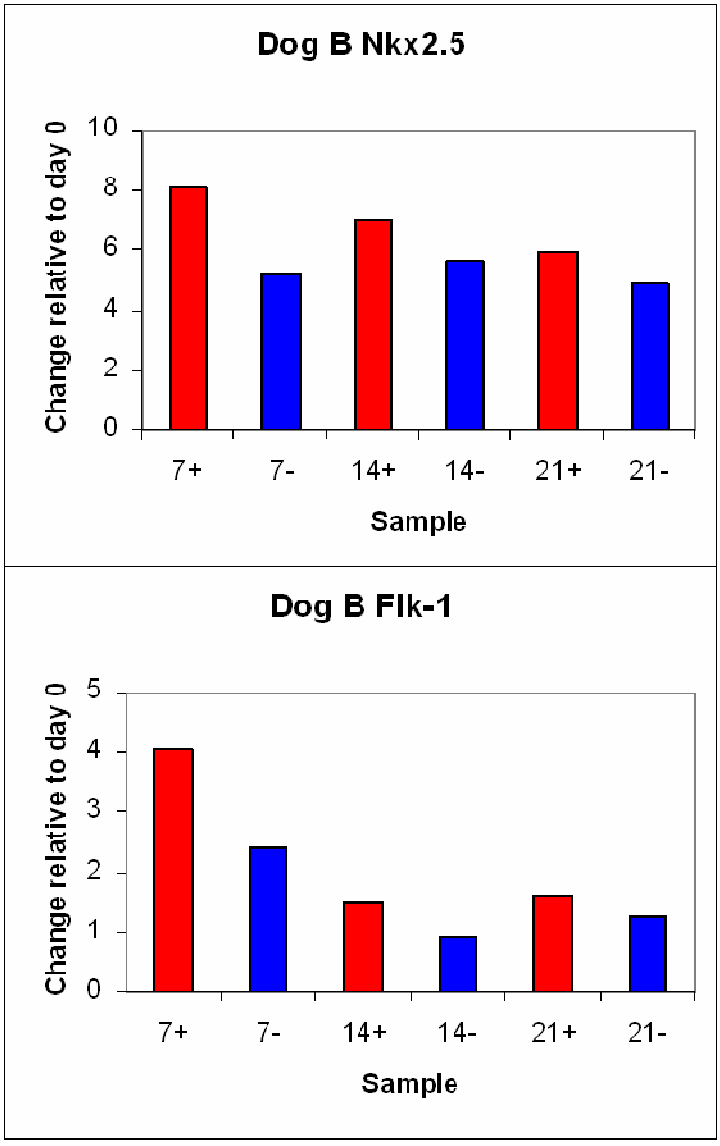
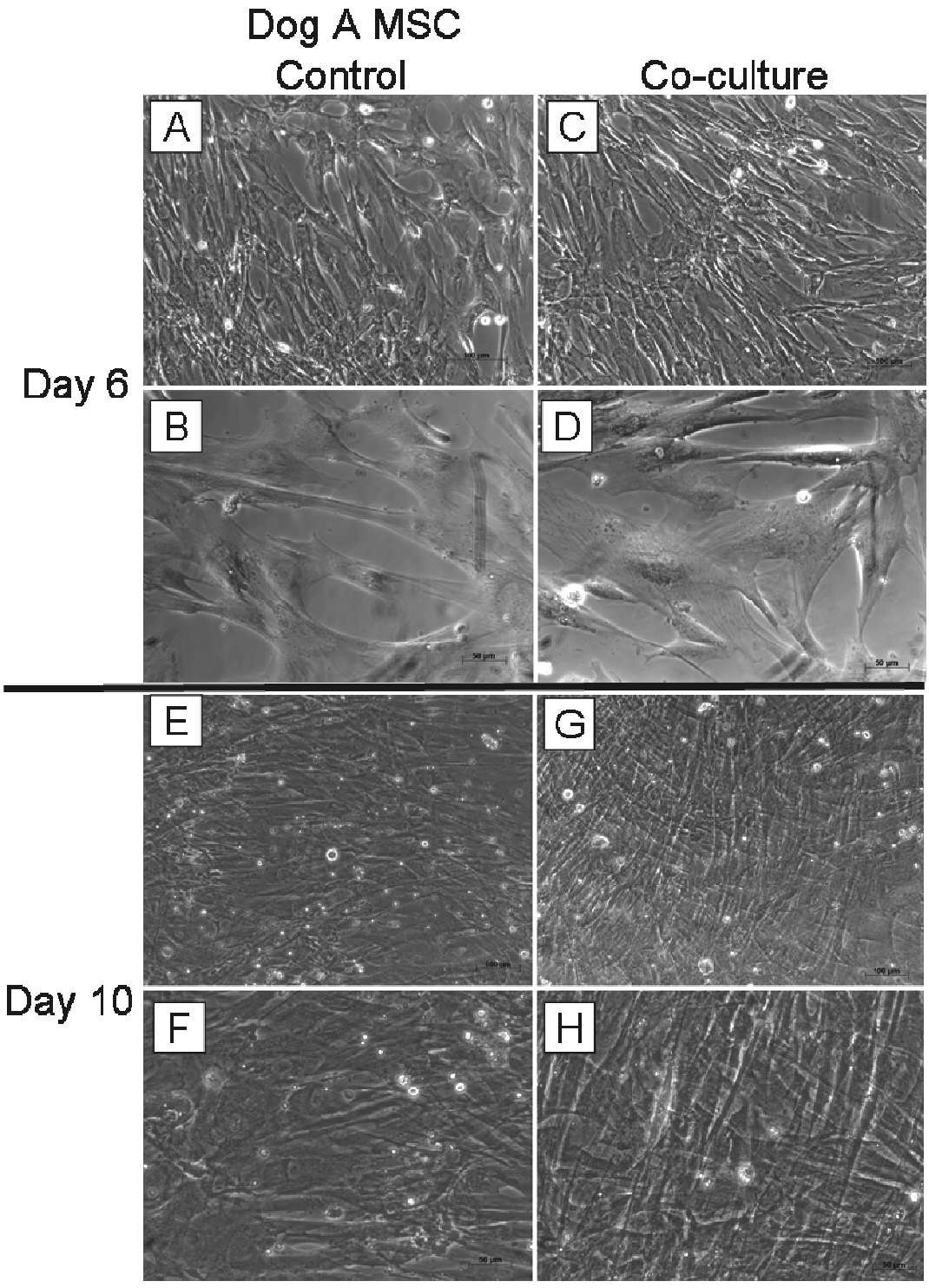


Figure 5.12. Quantitative RT-PCR analysis of dog B expression of Nkx2.5 and Flk-1 during cardiac directed differentiation relative to day 0. Test (+) and control (-) results are shown for days 7, 14 and 21.

5.3.3 Co-Culture of Mesenchymal Stem Cells with Cardiac Stem Cells

5.3.3.1 Cellular Morphology during Differentiation

Canine MSCs were co-cultured in a transwell system with autologous CSCs. The transwell inserts ensured that there was no direct cell to cell contact or cell fusion possible, thereby ensuring that only media and expressed factors were shared between the cell populations. Two separate dogs were run concurrently, A and B (these were not the same dogs used for the previous differentiation technique using 5'AZA). Morphologically the cells appeared to increase significantly in number over the time course of the experiment (Figure 5.13), and in particular dog B, which by day 6 showed a dense monolayer of cells in the control wells (Figure 5.13 Dog B day 6). Dog A co-culture wells appeared to have a very dense layer of cells by day 10, which formed cross-striation patterns in co-culture not seen in control wells (Figure 5.13, Dog A, G and H). During culture it was noted that dog B MSCs in a co-culture well peeled away from the gelatin coated well base by day 6 and formed a pellet of cells, which did not occur with dog A.


Dog A MSC
Control
Co-culture
A
C
Day 6
B
D
E
G
Day 10
F
H

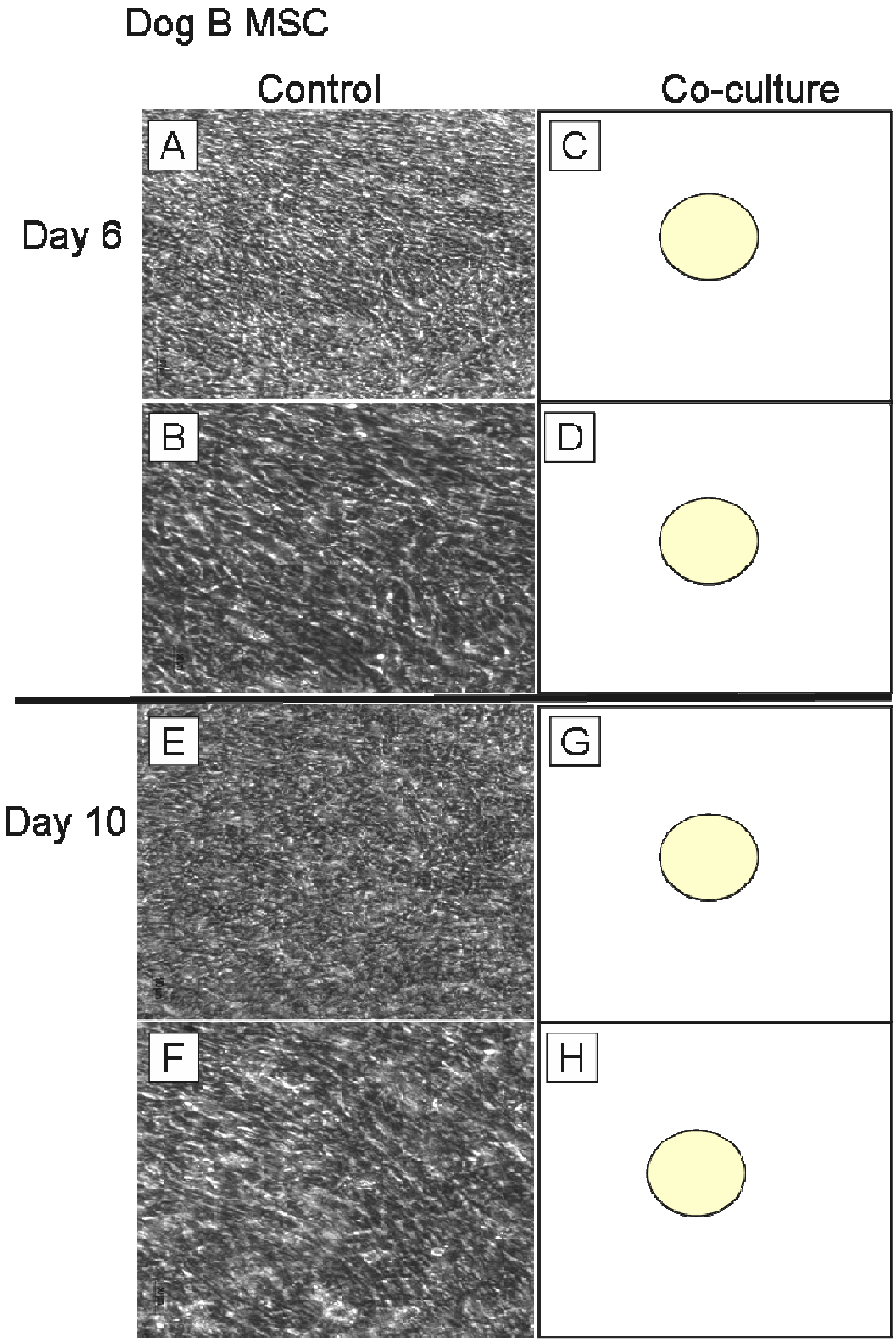

Dog B MSC
Control
Co-culture
A
C
Day 6
B
D
E
G
Day 10
F
H

Figure 5.13. Cellular morphology during co-culture of Canine MSCs with autologous CSCs. Canine MSCs showed minor morphological changes during co-culture compared to control wells at both time points for dog A , and cells peeling away into a pellet in co-culture wells of dog B (Magnification A, C, E, G x 100; B, D, F, H x 200).

5.3.3.2 Transcriptional Analysis of Mesenchymal Stem Cells during Indirect Co-Culture with Cardiac Stem Cells

Using semi-quantitative RT-PCR (Figure 5.14) cells from both dog A and B expressed CD44 at baseline and continued to express it at steady levels in both cell populations throughout the experiment. Dog A did not express CD34 throughout the experiment, however Dog B expressed CD34 at baseline, and clearly maintained expression in control wells at days 6 and 10, whereas in co-cultured wells expression was significantly lower at both time points. Islet 1 expression was seen at very low levels with dog A at all time points. Dog B also demonstrated low levels of islet 1 expression at baseline, day 6 control wells and day 10 co-culture wells, whereas day 6 co-culture did not express and day 10 control wells had much reduced expression. Nkx2.5 expression remained at similar high levels compared to baseline in dog A throughout the experiment. In dog B Nkx2.5 levels appeared to reduce in both control and co-culture wells at day 6, compared to day 0, and increase in control wells at day 10. Flk-1 expression remained relatively steady throughout the experiment for dog A, whereas for dog B there was a suggestion of reduced Flk-1 in both control and co-culture samples at day 6, and co-culture samples at day 10. VEGF expression remained steady throughout the

experiment for dog A, with multiple banding representative of splice variants. In dog B, there appeared to be a more pronounced upper band in co-culture wells at both time points when compared to baseline controls. vWF was not expressed by dog A, but there was low level expression in dog B, which appeared to be increasing with time in culture. SMA was steadily expressed throughout the experiment for both dogs (a dual band is seen representative of SMA above and GAPDH below, due to similar band sizes). Glomulin was steadily expressed in all wells in dog A and dog B except for a decrease in expression in co-culture wells at day 6 for dog B. Connexin 43 was expressed at steady levels for both dogs throughout the experiment. Cells from dog A and B were negative for CD45, β_1-adrenergic receptor, c-Kit, GATA 4, CTI, cardiac ryanodine receptor and CTT (Data not shown).

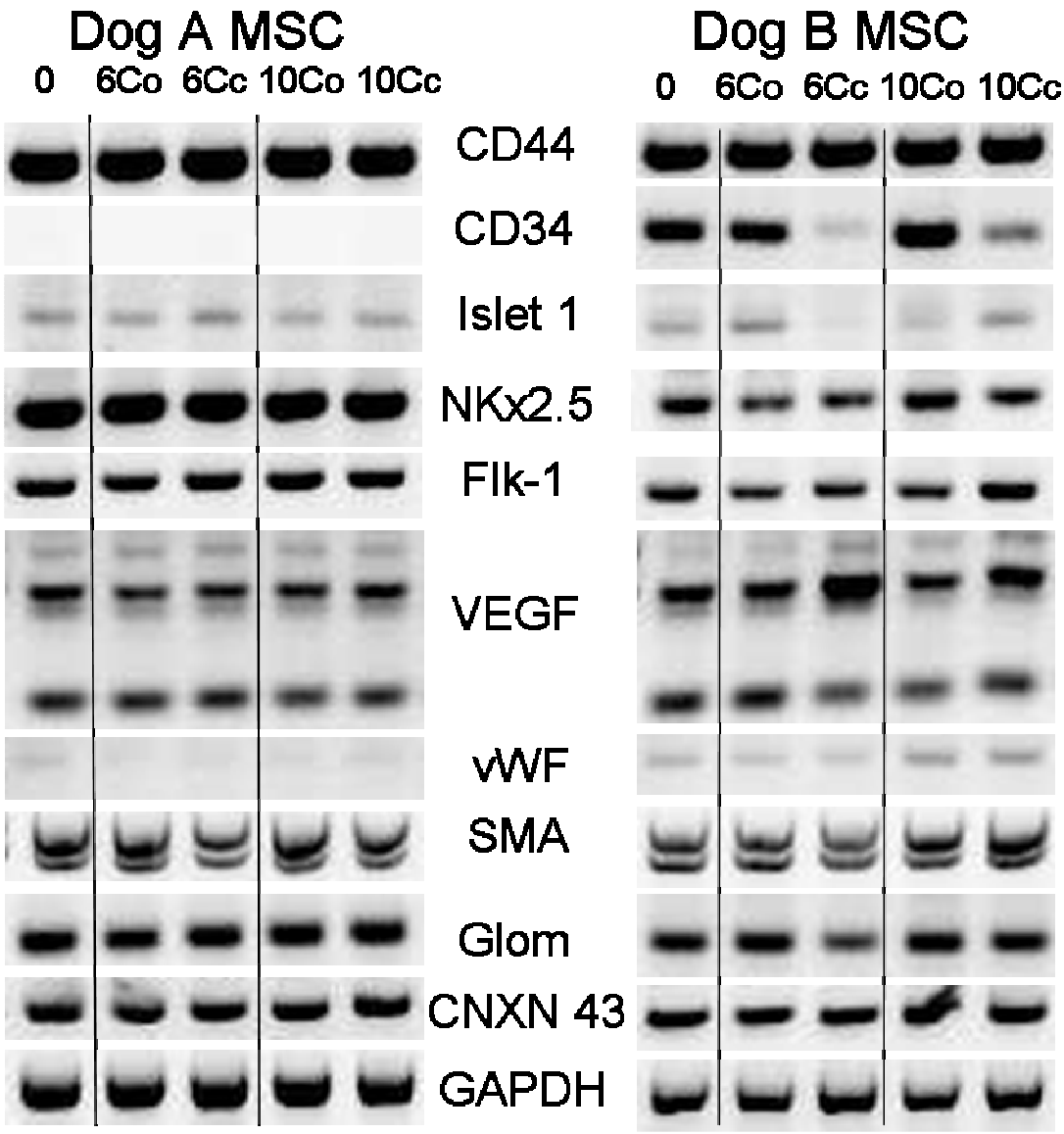


Figure 5.14. Semi-quantitative RT-PCR analysis of MSC expression during co-culture with autologous CSCs. Expression of each marker in control (Co) and co-cultured (Cc) wells at days 6 and 10 are compared to day 0 MSC baseline controls, and GAPDH. VEGF-vascular endothelial growth factor, vWF–von Willebrands factor, SMA–smooth muscle actin, Glom–glomulin, CNXN 43–connexin 43. GAPDH was run as a multiplexed positive control gene.

5.4 Discussion

MSCs offer great potential in the field of regenerative medicine due to their relative ease of handling in terms of isolation and culture, their apparent ability for multi-lineage differentiation and their potential in providing an autologous source of cells for regenerative therapies. Furthermore canine MSCs may offer a cell source to use for studying a number of diseases of great significance in the canine population. Cardiac disease, as previously discussed is the third biggest cause of mortality in pet pedigree dogs and development of an *in vitro* system for growing cardiomyocytes would greatly improve the opportunities for study. The behaviour of dog cells may also inform studies on other mammalian species most notably humans since they share living environments and develop similar acquired diseases resulting from a combination of genetic and environmental influences. In this study we aimed to isolate and characterise canine MSCs, and to evaluate their *in vitro* differentiation potential toward cardiac lineages using two techniques; addition of the de-methylating agent 5'AZA and indirect co-culture with autologous cardiac stem cells.

5.4.1 Can Canine Mesenchymal Stem Cells be Isolated and Cultured from the Bone Marrow?

This study demonstrates successful culture of primary bone marrow derived MSCs from the dog using a standardized technique (Dominici et al., 2006). This was reproducible in dogs of various ages, sex and breed. Cells were isolated using tissue culture plastic

adherence, a key determinant of MSC behaviour, and cell populations demonstrated log increases in cell number at passage once fully established.

Morphologically the cells resembled previously described MSC populations having a flattened, elongated appearance with multiple jagged projections (Pittenger and Martin, 2004, Shim et al., 2004). Canine MSCs would typically senesce between passage 4 and 6, at which point the cells would increase in size and the spindle projections would fatten. The canine MSCs closely resembled human MSC populations described in the literature which typically senesce relatively early under standard culture conditions (Banfi et al., 2000, Sekiya et al., 2002).

5.4.1.1 Characterisation of Canine Mesenchymal Stem Cells

No single marker is available to define a MSC and therefore panels of markers are generally employed, but panels used vary between publications (Dominici et al., 2006). This of course has lead to some variability in terms of characterisation based on marker expression and may allow for variability within cell populations isolated. A key marker expressed by MSCs is CD44, and our canine MSCs were positive for CD44 using both RT-PCR and IFA.

Despite the lack of a single marker to define MSC populations, STRO-1, a classic human MSC marker, has been used to enrich MSC populations from bone marrow isolates (Simmons and Torok-Storb, 1991, Gronthos et al., 1994, Dennis et al., 2002).

Canine MSCs were found to be STRO-1 positive using IFA, alluding further to similarities between canine and human MSC populations. STRO-1 is not available for use in rodent cells, a limitation in terms of cell comparisons.

Canine MSCs were CD45 and c-Kit low in early passage analysis and negative in later passage analysis using RT-PCR. This difference may reflect haematopoietic contamination in early analysed MSCs and is an unavoidable problem when isolating primary MSCs from bone marrow in the manner described; whereby any contaminating cellular expression is exponentially amplified in PCR to give low background result. Furthermore, the majority of canine MSCs isolated were CD34 negative, although one population was CD34 positive (seen analysed in co-culture experiments discussed below).

5.4.2 Can Canine Mesenchymal Stem Cells be Differentiated Toward Cardiac Lineages?

5.4.2.1 Cardiac Differentiation using Published Techniques

In this study we exposed primary canine MSCs to the 5'AZA differentiation technique described in the literature (Kruglyakov et al., 2006). We set criteria for cardiac differentiation based on behaviour in culture, including evidence of independent rhythmical beating and the expression of cardiac structural and functional genes.

We initially examined the morphology of the cells during the differentiation over a three week period. Morphologically there were some minor changes in appearance of the canine MSCs during cardiac directed differentiation. In differentiated wells, in particular at later time points there appeared to be some suggestion of lining up of the MSCs, which was not seen in the undifferentiated wells. This may represent a more organized cellular pattern, or may be a direct consequence of 5'AZA addition. In a functional cardiomyocyte the alignment of cells allows for the most efficient contraction, and is a critical factor in cardiac development. The lining up seen in the differentiated MSCs may have been the beginning of cardiomyocyte spatial patterning.

We then examined the differentiated and undifferentiated cells at all time points for marker expression. We developed a bespoke panel of markers representing those expressed by MSCs, cardiac cell lineages, and those expressed by known stem cell populations. Specifically this panel of markers included markers which have been used to define successful cardiac differentiation in previous work (Table 5.8).

Table 5.8. Markers expressed in MSC populations following cardiac differentiation

Author	*Species*	*Markers expressed in differentiated MSCs*
Krugalykov et al	Rat	GATA 4, Nkx2.5, CXN43, CTT, CTI, Cardiotrophin 1
Xu et al	Human	β-MHC, desmin, α-cardiac actin, CTT
Shim et al	Human	CTI, desmin, GATA 4, Nkx2.5, CTT
Antonitisis et al	Human	vimentin, α-cardiac actin, β-MHC, CTT
Makino et al	Mouse	GATA 4, Nkx2.5
Fukuda et al	Mouse	MEF2A, MEF2D

(Makino et al., 1999, Shim et al., 2004, Xu et al., 2004, Kruglyakov et al., 2006, Antonitsis et al., 2007).

All of the markers examined during cardiac directed differentiation generally maintained expression levels with two exceptions; Nkx2.5, a cardiac homeobox gene, and Flk-1, an endothelial marker. This suggests that there was minimal effect of 5'AZA upon the MSCs in driving the cells towards a cardiac lineage, particularly given the lack of expression of cardiac structural protein genes. Nkx2.5 and Flk-1 have both been used to define cardiac stem cells (Beltrami et al., 2003, Goumans et al., 2007, Yang et al., 2008) and were included in the panel to allow for comparisons to that cell source. Some variability was seen in expression of Nkx2.5 and Flk-1 between the two dogs examined, however this is to be expected given the primary source of the cells and individual dog variation. The general trend in both markers was increased expression in differentiated cells, however there was not a striking difference, and both markers were expressed in

day 0 baseline MSCs. Nkx2.5 is an primitive cardiac marker (Lints et al., 1993) and Flk-1 a primitive endothelial marker (Yamaguchi et al., 1993) and the expression of both of these in MSCs may be due to the combined mesodermal lineage of the cell types. The variation in Nkx2.5 and Flk-1 expression is likely to have been driven by the addition of the demethylating agent 5'AZA, increasing transcription of these markers and this increase has been described in previous studies using 5'AZA for cardiac differentiation (Makino et al., 1999, Kruglyakov et al., 2006). In comparison to our isolated canine CSCs, Nkx2.5 was seen to increase similarly, however CSCs decreased Flk-1 expression following cardiac differentiation. This interesting difference may be representative of movement of the CSCs towards cardiomyocytes and MSCs towards a more endothelial cell type.

Based on the marker expression and morphology following cardiac differentiation of canine MSCs no conclusive evidence of cardiac differentiation following 5'AZA differentiation protocols was seen. Interestingly there is some evidence to suggest that differentiation ability of MSCs varies amongst MSC populations and those isolated from different regions of the body (Kern et al., 2006, Liu et al., 2007, Vidal et al., 2008). There is no evidence to discuss this for cardiac differentiation, but it has been noted for connective tissue differentiation (discussed in Chapter 7) and this may be relevant to this experiment, and should be investigated further by performing cardiac differentiation using MSCs from different sources.

5.4.2.2 Differentiation of Canine Mesenchymal Stem Cells using Indirect Co-Culture

Stem cell co-culture has commonly been used as a method for triggering differentiation of cell populations, in particular MSCs co-cultured with cardiomyocytes either indirectly, or directly can drive MSC differentiation toward the cardiac lineage (Wang et al., 2006, Koninckx et al., 2009). Furthermore, it has been shown that MSCs can move toward cardiac lineages when cultured in cardiomyocyte conditioned media in hypoxic conditions (Xie et al., 2006). MSCs have also been found able to drive cardiac differentiation themselves both in embryonic stem cells and cardiac stem cells (Hatzistergos et al., 2010, Yue et al., 2010). With these findings in mind, a novel indirect co-culture experiment using autologous canine MSCs and CSCs was established.

Initial examination of morphology indicated some changes both between control and co-culture wells, but also between dogs. Dog A varied little in culture morphologically, but subjectively there was suggestion of cross-striation of cells in co-culture wells by day 10 not seen in control wells. Dog B MSCs in co-cultured wells peeled away from the base of the well by day 6 and grew in a 3D pellet, which was not seen in the control wells. This peeling away is curious, as it did not occur in the control wells, and both wells received the same number of initial cells. The peeling away may be a reflection of increased cell replication with confluence being reached rapidly, or perhaps of strong cell-cell bonds forming and subsequent cellular contraction. It would be safe to conclude that either way there was an influence from the co-cultured CSCs given the peeling did

not occur in control wells, and this may be due to released soluble factors from the CSCs which the MSCs responded to.

Using transcript analysis it became apparent that there was little difference in transcript expression during co-culture in both dogs. No cardiac lineage markers were expressed, and there was no beating or obvious morphological changes to suggest successful cardiac differentiation.

Of note in this experiment was the difference in starting population of cells from dogs A and B at baseline. Dog B was CD34 positive, which was not seen in dog A cells and critically does imply that comparisons between A and B are difficult given they are potentially separate populations of cells. CD34 is generally believed to be negative in MSC populations (Dominici et al., 2006) and had not been found expressed in previous canine MSCs that we isolated. CD34 appears to function as a cell surface adhesion molecule (Nielsen and McNagny, 2008) but may also play a role in angiogenesis (Copland et al., 2008). Human MSCs have been described as both positive (Shim et al., 2004) and negative for CD34 (Pittenger et al., 1999, Xu et al., 2004, Antonitsis et al., 2007). Kim *et al* found that human MSCs were negative for CD34 antibody using FACS analysis, but found the cells expressed CD34 using RT-PCR (Kim et al., 2005a). This variation in CD34 expression seen in both human MSCs and the dog MSCs in this study may simply be technique dependent but may reflect cell heterogeneity, or importantly indicate a subpopulation of MSCs, which express both mesenchymal, and haematopoietic markers. The decrease in CD34 expression seen in the co-cultured cells

may be due to spatial differences in culture, given that the cells formed a pellet which may decrease CD34 expression, but may also be due to factors expressed by CSCs which influenced CD34 expression in these cells. This effect will need to be examined further to elucidate the precise mechanism behind CD34 downregulation.

5.4.2.3 Differentiation of Mesenchymal Stem Cells and their Role in Cardiac Disease

It can be seen from our experiments that cardiac directed differentiation of primary canine MSCs was unsuccessful. Differentiation of MSCs *in vitro* is a contentious issue; alternative literature describing co-culture experiments and 5'AZA differentiation have shown that differentiated cells demonstrated no electrophysiological properties of cardiomyocytes. Furthermore the efficiency of differentiation was seen to be very low at approximately 0.07% (Martin-Rendon et al., 2008, Rose et al., 2008). This demonstrates the conflicting evidence within this field, and that accurately defining differentiation using cellular morphology and marker expression is controversial.

Even though cardiac differentiation was unsuccessful and therefore limits the use of MSCs to study cardiac diseases *in vitro,* it does not preclude the use of MSCs for cardiac disease. As we have shown, the CD34 positive MSCs down-regulated CD34 in response to co-culture with CSCs, and also altered their VEGF banding expression which may represent cytokine release and cellular proliferation associated with the paracrine effects of MSCs. It has been shown that MSCs can survive in the damaged heart for extended

periods of time (Stuckey et al., 2006) yet it is unknown if MSCs transform into cardiomyocytes *in vivo*, undergo cell fusion giving rise to cells phenotypically similar to cardiomyocytes (Nygren et al., 2004), or act in a paracrine fashion modulating immune responses (Meirelles Lda et al., 2009). MSCs may exert a paracrine influence in multiple ways, including trophic, immunomodulatory, anti-fibrotic and chemotactic effects and large numbers of secreted molecules are thought to be involved in these pathways. *In vitro* MSC conditioned media protects against apoptosis induced by hypoxia increasing both proliferation of cardiac stem cells and expression of cardiac specific markers such as β-MHC, c-Kit and atrial natriuretic peptide in rat CSCs (Nakanishi et al., 2008, Yu et al., 2009b). Furthermore MSCs exert an influence upon the Wnt pathway by secreting antagonists of canonical Wnt ligands following myocardial damage leading to up-regulation of anti-apoptotic genes (Mirotsou et al., 2007, He et al., 2011). Therefore the paracrine effects of canine MSCs and their role in cardiac disease is an exciting area of future research.

5.4.3 VEGF Splice Variation and Significance to Mesenchymal Stem Cells?

VEGF was seen in multiple bands representative of its several splice variants throughout all experiments, and there was no significant alteration in banding expression between differentiated cells and those of day 0 MSCs in 5'AZA differentiation. The lack of general banding change in the majority of the MSCs reinforces the conclusion that cardiac directed differentiation was unsuccessful, given the alternative banding pattern seen in differentiated whole heart tissue which was never seen in any MSC population

(see Chapter 4; Cardiac Stem Cells). However, there was an increase in the expression of the upper band of VEGF corresponding to $VEGF_{182/188}$ in co-cultured wells of dog B during in-direct co-culture which was intriguing. VEGF isoforms $VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$ each have similar yet individual effects. All three promote MSC proliferation with $VEGF_{120}$ and $VGEF_{188}$ associated with increased expression of growth factors and cytokines and $VEGF_{164}$ promoting genes associated with remodeling and endothelial differentiation (Lin et al., 2008). It is likely that the increase in the upper band in dog B MSCs in co-cultured wells is representative of an upregulation of $VEGF_{182/188}$ and thus growth factor and cytokine expression release in response to being in the co-culture environment which may have played a part in the down-regulation seen in CD34 expression.

5.4.4 Conclusion

This study demonstrates a number of interesting findings. Firstly, canine MSCs can be isolated from the bone marrow using traditional techniques and compare favourably with human MSCs. Secondly, dog MSCs are not capable of differentiation into functional cardiomyocytes, demonstrated using two separate techniques and the differentiation criteria set out in this study. This study indicates the need for further work in identifying factors which affect efficiency of cardiac differentiation, and may warrant more global marker characterisation of individual MSC populations given the variety seen in baseline marker expression. Furthermore, this study has highlighted the need for further analysis of the paracrine effects of MSCs and the role these may have in cardiac disease therapy.

CHAPTER 6

Mouse Mesenchymal Stem Cells and the Cardiovascular System

Abstract

Based upon the results obtained in Chapter 5 it was deemed relevant to obtain an MSC source from an alternative species which had successful cardiac directed differentiation described in the literature. Murine MSCs are a readily available source of adult stem cells enabling extensive *in vitro* study of this cell population. Furthermore, due to the availability of mouse models, *in vivo* studies utilizing MSCs are widely used. MSCs have been described as multipotent, and have been proven capable of differentiation into several connective tissue types. Furthermore some studies have suggested an ability to differentiate into non-connective tissue cell types such as cardiomyocytes. The aim of this study was to differentiate murine MSCs towards cardiac lineage to allow for comparison with canine MSCs using culture with 5' Azacytidine. Critically, baseline analysis of gene expression of passage four MSCs demonstrated expression of key cardiac markers including cardiac troponin T and I, and the ryanodine receptor. Furthermore, expression analysis of these genes changed with time in culture and passage number with no significant alteration when subjected to a cardiac differentiation

protocol. This study therefore highlights the importance of characterizing the undifferentiated cell population extensively, and indicates the limitations in extrapolating data for comparison between species.

6.1 Introduction

Mesenchymal stem cells (MSCs) are defined using three key parameters as discussed in Chapter 5; Mesenchymal Stem Cells and the Cardiovascular System. In brief they must adhere to tissue culture plastic, express specific surface antigens and be capable of multipotent differentiation *in vitro* (Dominici et al., 2006). In recent years, aided by ease of isolation and culture, this cell population from the mouse has been extensively studied in relation to use of these cells as a model for *in vitro* and *in vivo* MSC research including differentiation potential and their use as a therapeutic agent and a source of cellular precursors for tissue regeneration.

6.1.1 Differentiation Potential of Mouse Mesenchymal Stem Cells

Murine MSCs are capable of differentiation along three standard routes namely osteoblast, adipocyte, and chondrocyte cell types (Phinney et al., 1999, Meirelles Lda and Nardi, 2003, Tropel et al., 2004). Furthermore there is a suggestion that these mesodermal lineage cells have the ability to differentiate into non-connective tissue cell types such as neuronal, endodermal and cardiomyocyte (Kopen et al., 1999, Orlic et al., 2001a, Jiang et al., 2002).

6.1.1.1 Differentiation of Murine Mesenchymal Stem Cells toward Cardiac Lineages

6.1.1.1.1 *In Vivo* Differentiation

It has been suggested that murine MSCs are capable of differentiating into cardiomyocytes *in vivo* when injected into the border zone of artificially created infarcts in mice and may contribute to improved repair of the infarcted area (Orlic et al., 2001a). Furthermore, murine MSCs have been found to translocate to damaged myocardium from the bone marrow in response to injury. Orlic *et al* described the relocation of murine c-Kit positive MSCs from the bone marrow to the damaged myocardium following induced myocardial infarction and it was proposed that this cell movement was in response to cytokines, including granulocyte colony stimulating factor (G-CSF) and stem cell factor (KitL) (Orlic et al., 2001b). More recently it has been found that mobilized c-Kit/Flk-1 positive cells from the bone marrow relocate to damaged myocardium and improve myocardial repair by promoting angiogenesis by increasing VEGF expression (Fazel et al., 2006). Therefore it is likely that MSCs play multiple roles in the damaged heart; there is a suggestion that MSCs differentiate to functional cardiomyocytes, which may incorporate into damaged tissue to replace lost cells, but there is also evidence that MSCs affect repair by paracrine means, improving vascularisation of damaged areas. These systems need further investigation to determine the pathways involved, and the development of *in vitro* MSC cardiac differentiation has been the first step towards allowing further research in this area.

6.1.1.1.2 *In Vitro* Differentiation

Murine MSCs have been described as being able to differentiate into cardiac tissue *in vitro* using the de-methylating agent 5'AZA. Bone marrow derived MSCs positive for GATA 4, Nkx2.5 and MEF2C prior to differentiation were exposed to 3 μmol/L 5'AZA for 24 hours which after 4 weeks induced spontaneously beating cells positive for sarcomeric myosin, actinin and desmin (Makino et al., 1999, Fukuda, 2001). A variation in the 5'AZA technique was used by Kruglyakov *et al* whereby bone marrow derived GATA 4 and Nkx2.5 positive MSCs from the rat were exposed to 3 μmol/L 5'AZA every 3 days for 21 days to initiate cardiac directed differentiation. This resulted in an increase in cardiac troponin I and T and connexin 43 suggestive of successful cardiac differentiation (Kruglyakov et al., 2006). These *in vitro* derived cardiomyocytes may be used for the investigation of the role of MSCs within the damaged heart; for instance investigating the mechanisms of cardiac differentiation and the growth factors and cytokines which may be released in response to damage. However, the ability of MSCs to differentiate toward cardiomyocytes *in vitro* is controversial. Cardiac differentiation *in vitro* is defined using marker expression and evidence of beating as has been previously discussed (Chapter 5; Canine MSCs and the Cardiovascular System), but there is a significant variation in the markers used to determine successful MSC differentiation. Furthermore, there are questions regarding the MSC marker profile prior to differentiation. Mouse MSCs have been found to express cardiac markers, such as cardiac troponin T and I at baseline which indicates an MSC population with

cardiogenic potential but therefore makes it more difficult to define successful differentiation based upon cardiac marker expression (Rose et al., 2008).

6.1.2 Marker Variation Between Murine and Human Mesenchymal Stem Cells

Rodent modeling is commonly used to investigate MSC properties and characteristics, however characterization of MSC populations is challenging given the wealth of markers available (Kolf et al., 2007). When reviewing the literature differences between human and murine MSC populations become readily apparent (Table 6.1).

Table 6.1. Marker expression in mouse and human MSCs.

Surface Antigen	*Human*	*Mouse*
STRO-1	+	-
CD29	+	+
CD44	+	+
CD106	+	+
CD11b	-	-
CD31	+/-	-
CD34	+/-	+/-
CD45	-	-
C-Kit	-	+/-
Sca 1	-	+
CD90	+	-
Flk-1	+/-	-

Human; STRO-1 (Simmons and Torok-Storb, 1991, Dennis et al., 2002); CD29, CD44, CD106, CD90 (Pittenger et al., 1999); CD44, CD11b, CD31, c-Kit, CD90, Flk-1 (Colter et al., 2001); CD34, CD45 (Etheridge et al., 2004, Shim et al., 2004).

Mouse; CD44, CD29, CD106, CD11b, CD31, CD45, c-Kit, Sca 1 (Baddoo et al., 2003); CD29, CD44, Cd11b, CD31, CD45, Sca 1 (Meirelles Lda and Nardi, 2003); CD29, CD44, CD11b, CD31, c-Kit, CD90 (da Silva Meirelles et al., 2006); CD11b, CD31, CD34, CD90, Flk-1 (Peister et al., 2004); c-Kit (Orlic et al., 2001a).

Marker variation has led to difficulties in making comparisons of MSCs between different species, such as between mouse and human. Variation is also seen in MSC populations isolated from within a single species, which has led to further confusion within the field (Peister et al., 2004). This variation in marker expression may represent different MSC populations, each with their own properties and differentiation potential, but may also represent a single MSC population at different stages of development and cellular expression. Characterising MSCs extensively prior to experimentation is therefore critical when analyzing subsequent results, such as in differentiation experiments.

6.1.3 Aims

The aim of this chapter is to use mouse MSCs as a model for *in vitro* cardiac differentiation for canine MSCs based upon the literature claiming successful *in vitro* differentiation and the work performed in Chapter 5; Canine Mesenchymal Stem Cells and the Cardiovascular System. Cell morphology and extensive baseline marker characterization will be performed on the mouse MSCs, followed by cardiac directed differentiation. The differentiated cells will then be analysed using transcriptional analyses and compared to the canine results.

6.2 Materials and Methods

6.2.1 Murine Mesenchymal Stem Cell Culture

6.2.1.1 Isolation and Culture of Primary Murine Mesenchymal Stem Cells

Four 34 day old male entire C57/Bl6 mice were euthanised humanely using a schedule 1 procedure and donated to this project. The diaphyses of the femurs and tibias of each mouse were flushed into heparinised MSC media (as described in Chapter 5), with pooling of each bone source per mouse. Cell suspensions were made up to 10 mL using standard MSC media. 16 mL of Ficoll Paque Premium (GE Healthcare, UK) was loaded into the base of a 50 mL Falcon tube. Cell suspension was gently overlaid onto the surface of the Ficoll, and then the Falcon tubes were centrifuged at 450g for 30 minutes without a brake at room temperature. Following centrifugation, the interface layer was gently aspirated and transferred to a 25 mL Falcon tube and made up to 10 mL with PBS. The cells were then centrifuged at 150g for 5 minutes at room temperature, and the PBS removed and the cells washed with 7 mL of standard MSC media. The cells were again centrifuged for 5 minutes at 150g, and the pellet subsequently resuspended in 1 mL of standard MSC media. Cells were counted using the haemocytometer and white cell counting mount fluid (1% w/v crystal violet and 2% v/v glacial acetic acid in distilled water). Cell count for each mouse ranged from approximately 9×10^5 to 2.5×10^6 cells total. These cells were plated into T25s, thereby giving a final cell density of 3.6×10^4 cells/cm^2 to 1×10^5 cells/cm^2. Media changes were performed on the cells

twice weekly. At 2 weeks post harvest the cells were passaged into new T25s. Cells were washed using PBS and 0.05% Trypsin-EDTA was applied to the cells and incubated at 37 °C, 5% CO_2 for 5-10 minutes to release the cell attachments. Cell suspension was aspirated from the flasks, and 5 mL of standard MSC media added to the mix. Cells were then centrifuged at 1200 rpm for 5 minutes to pellet, and cells resuspended in 2 mL of standard MSC media. Cell suspensions were then directly transferred to new T25 flasks and replaced at 37 °C, 5% CO_2. At 4-5 weeks post harvest cells had stopped growing and evidence of cell death was seen, therefore flasks were discarded.

6.2.1.2 Commercially Available Murine Mesenchymal Stem Cell Culture

Murine MSCs were purchased from Invitrogen (Cat. No. 510502-01) and thawed from liquid nitrogen storage at 37 °C in a water bath for up to 2 minutes. 10 mL murine MSC media; DMEM low glucose containing Glutamax-I (Invitrogen), with 10% MSC qualified FBS (Invitrogen Cat. No. 12662-011) and 100 U/mL penicillin G and 100 μg/mL streptomycin (both Invitrogen) was added in a slow drop-wise fashion. The cells were then centrifuged for 5 minutes at 300g at room temperature. The cell number was calculated and cells were plated at a cell density of 5,000 cells per cm^2 in a T75 at 37 °C, 5% CO_2 in murine MSC media. Cells were passaged every 3-4 days, and no evidence of senescence was observed.

6.2.1.2.1 Culture of Murine Mesenchymal Stem Cells in Murine and Standard (Canine) Media

Following initial culture in murine MSC media (containing MSC qualified FBS) 1.25 x 10^5 murine cells were transferred into 7 mL standard MSC media (containing basic FBS, previously used for Canine MSC work, Chapter 5; Canine Mesenchymal Stem Cells and the Cardiovascular System) at second passage in a T25. A subculture of murine MSCs was then run in tandem in each media. Media changes were performed every 3/4 days, and cells were passaged at 80-90% confluency (approximately twice a week). 1.25 x 10^5 cells were replaced into a T25 at each passage. A harvest of cells were pelleted at passage 4 (post-revival) and at passage 18. These cells were snap frozen and RNA subsequently extracted and cleaned up using DNase 1. RNA was quantified using the Thermo Scientific Nanodrop ™ machine and 500 ng of RNA was reverse transcribed using Omniscript RT kit and the cDNA stored at -20 °C. cDNA was used for PCR reactions to compare between passage 4 and 18 for murine MSCs cultured in both murine MSC media and standard (canine) media using primers designed using the murine genome and primer 3 software (Table 6.2). PCR reactions were analysed using agarose gel electrophoresis and GAPDH was used as a housekeeping control gene in a separate reaction.

6.2.2 Characterisation of Murine Mesenchymal Stem Cells

6.2.2.1 Characterisation of Murine Mesenchymal Stem Cells using RT-PCR

Murine MSCs were analysed during cardiac differentiation for all markers described in Table 6.2 using semi-quantitative RT-PCR, as described below.

6.2.2.2 Characterisation of Murine Mesenchymal Stem Cells using IFA

2 x 10^4 murine MSCs were added to each well of a 4 well chambered slide at passage 4. These cells were incubated for 48 hours at 37 °C, 5% CO_2 at which point the cells had adhered to the slides and increased to approximately 50% confluency. Slides were washed and were fixed as described in Chapter 2; Materials and Methods. Primary antibody rabbit polyclonal to CD44 – C terminal (Abcam, UK. Catalogue Number; Ab65829) was added to the slides at 1:200, diluted in PBST (0.1% normal goat serum; 0.1% Tween 20 in PBS) and left overnight in a humidified chamber at 4 °C. The following day the cells were washed, and labeled with secondary antibody Alexafluor® 488 conjugated goat polyclonal anti-rabbit IgG (Invitrogen, UK. Catalogue number A-11034), at 1:500, for 1 hour at room temperature. Nuclei were subsequently labeled using a DAPI mount (Vectastain, Vector Labs, UK) and slides stored at -20 °C. Slides were viewed and imaged using the Axiovision programme (Carl Zeiss International).

6.2.3 Mouse Mesenchymal Stem Cell Differentiation to Cardiac Lineages

6.2.3.1 Cardiac Differentiation Technique

Mouse MSCs were differentiated toward a cardiac lineage using the same technique as described for canine MSCs (Chapter 5; Canine Mesenchymal Stem Cells and the Cardiovascular System). Murine MSCs were differentiated in both standard canine MSC media (containing basic FBS) and murine MSC media (containing MSC qualified FBS). At set up a pellet of day 0 cells was snap frozen as control cells. During differentiation harvests of cells were taken at day 7, 14 and 21 as previously described and cell pellets snap frozen and all cell pellets were stored at -80 °C.

6.2.3.2 RNA Extraction and Reverse Transcription

RNA extraction and reverse transcription for semi-quantitative RT-PCR was performed as described in Chapter 2; Materials and Methods. In brief, RNA was extracted using the RNeasy® mini kit and cleaned up with DNase 1. 100 ng of RNA was reverse transcribed using the Omniscript RT kit, and subsequent cDNA stored at -20 °C.

6.2.3.3 Polymerase Chain Reaction

cDNA from the reverse transcription step was used for PCR to analyse for marker expression. Primers for each marker were designed specifically for the mouse (Table

6.2), and where possible were intron spanning. All amplicons were subsequently sequenced to confirm primer specificity.

Table 6.2. Murine oligonucleotides. CTT-Cardiac Troponin T; Beta 1 Ad-β_1-adrenergic receptor; Cardiac RyR-Cardiac Ryanodine Receptor; CTI-Cardiac Troponin I; VEGF-Vascular Endothelial Growth Factor; vWF-Von Willebrands Factor; SMA-Smooth Muscle Actin. F: Forward Primer. R: Reverse Primer

Marker	Sequence 5'-3'	Size (BP)	Tm (°C)
C-Kit	F: AGG GAT TCC CGG AGC CCA CA	252	65
	R: GGG CCT GGA TTT GCT CTT TAA ATG C		
Islet 1	F: GGT TTC TCC GGA TTT GGA AT	183	57
	R: CAC GAA GTC GTT CTT GCT GA		
NKx2.5	F: GTG AAA CCT GCG TCG CCA CCA T	469	59
	R: TAG ACC TGC GCC TGC GAG AAG A		
GATA 4	F: CAA GAT GAA TGG CAT CAA CC	216	57
	R: GGT TTG AAT CCC CTC TTT CC		
Flk-1	F: AAG GCG CTG CTA GCT GTC GC	164	65
	R: TCC CGC TGT CCC CTG CAA GT		
CTT	F: TGT CCA ACA TGA TGC ACT TTG GAG G	165	65
	R: GCT CCT TGG CCT TCT CTC TCA GT		
Beta 1 Ad	F: TCC TTC TAC GTG CCC CTG TGC A	440	59
	R: CGC TGG AAA GCC TTG CGA AGT		
Cardiac RyR	F: GTT CTG CAG TGC ACG GCG ACC	268	66
	R: GGC CTC CAC CTT GAG CAG TCT TCA T		
CTI	F: CAG CGA TGC GGC TGG GGA AC	297	66
	R: CGA GCG TGA AGC TGT CGG CA		

Marker	Sequence 5'-3'	Size (bp)	Tm (°C)
VEGF	F: GTG CAC TGG ACC CTG GCT TT	300	62
	R: CCG CAT GAT CTG CAT GGT GAT GT		
vWF	F: GGG TCT GCA ACT GCC CAC CC	160	66
	R: GTG GGG CCC AAT GTT GGC CT		
SMA	F: GCC CAG CCA GTC GCT GTC AG	180	66
	R: TTA CTC CCT GAT GTC TGG GAC GTC C		
CD44	F: AGC ACC TTG GCC ACC ACT CCT A	306	59
	R: AGC TGC AGT AGG CTG AAG GGT T		
CD34	F: TGG CCC AGG GTA TCT GCC TGG	212	66
	R: GCT GGG AAG TTC TGT GCT ATT GGC C		
CD45	F: CCT TAC CTG CTC GCA CCA CTG AA	419	59
	R: GCT TGC AAG GCC CAG AGT GGA T		
Connexin 43	F: ATG AGC AGT CTG CCT TTC GT	249	58
	R: TCT GCT TCA AGT GCA TGT CC		
GAPDH	F: CAT CAA CGG GAA GTC CAT CT	428	Various
	R: GTG GAA GCA GGG ATG ATG TT		

A master mix for all primers was prepared using the Promega Go-Taq PCR kit (Table 6.3):

Table 6.3. Target gene master mix for PCR.

Master Mix Component	***1 x (µL)***
DEPC Treated Water	32.25
dNTP	1
$MgCl_2$	4
5 x GoTaq Buffer	10
Target Forward Primer	0.25
Target Reverse Primer	0.25
GoTaq polymerase	0.25

48 µL of PCR master mix was added to 2 µL of sample cDNA in a PCR reaction tube (Axygen, USA). Reactions were run as described in Chapter 2; Materials and Methods. Gene target primers were run at a final concentration of 0.5 pmol/µL with GAPDH run as a separate sample. Negative controls of RNA and water were run alongside to confirm absence of genomic DNA contamination. PCR results were analysed using agarose gel electrophoresis as previously described in Chapter 2; Materials and Methods.

6.3 Results

6.3.1 Murine Mesenchymal Stem Cell Isolation and Culture

6.3.1.1 Primary Mesenchymal Stem Cell Isolation and Culture

Murine MSCs were isolated from femurs of male C57/Bl6 mice which were approximately 34 days old. Cells were plated as described and maintained in standard MSC media. Media changes were performed twice weekly, and at first media change a significant number of floating cells and debris was noted. After 2 weeks of culture it appeared cells had senesced, and were therefore passaged to try and revive growth. Live cells were seen following passage, and 1 week post passage cells were starting to enlarge in size and spread out resembling MSCs isolated from the dog. Regular media changes were performed; however by 1 month post harvest cells were dying, and significant debris was seen in media. By 5 weeks post harvest all flasks from the 4 mice were discarded due to cell death and poor growth.

6.3.1.2 Commercially Available Murine Mesenchymal Stem Cell Culture

Purchased murine MSCs were cultured as described in Materials and Methods above. Morphologically murine MSCs had a flattened adherent shape, with jagged projections (Figure 6.1). These cells were capable of tripling cell number when passaged every 3-4 days. This continued through to passage 18, when experiments were stopped, with no

evidence of senescence. No difference was seen in cell morphology in cells cultured in either murine MSC media or standard media (Figure 6.2).

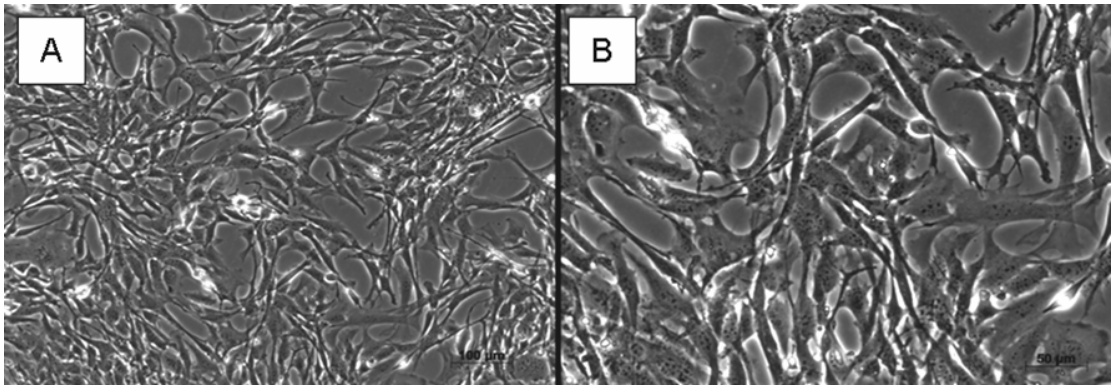


Figure 6.1. Purchased murine MSCs in culture. MSCs displayed typical morphology, with long flattened cells with multiple jagged projections. A-magnification x 100, B-magnification x 200.

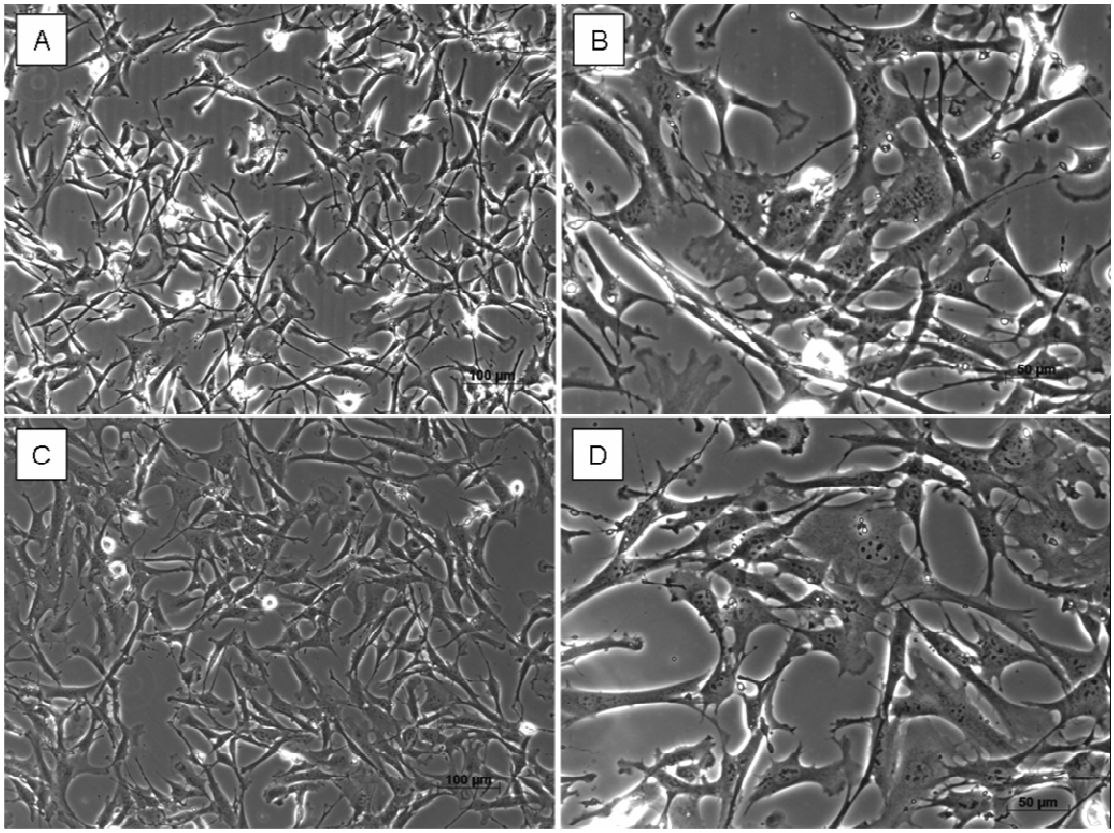


Figure 6.2. Purchased murine MSC cultures in mouse MSC media (A and B) and standard (canine) MSC media (C and D). Images taken at passage 4, 3 days post passage. A and C magnification x 100, B and D magnification x 200.

6.3.2 Characterisation of Murine Mesenchymal Stem Cells

6.3.2.1 Characterisation of Murine Mesenchymal Stem Cells using RT-PCR

Murine MSCs were analysed at day 0 during the cardiac differentiation experiment (see Figure 6.5), giving a full profile of baseline MSC expression. Murine MSCs expressed CD44 and CD34, and also expressed Nkx2.5 and VEGF at high levels. Low level

expression of GATA 4, Flk-1, CTI, RyR and connexin 43 was also seen. Baseline MSCs did not express Islet 1, β_1-adrenergic receptor, c-Kit, CD45 and CTT.

6.3.2.2 Characterisation of Murine Mesenchymal Stem Cells using IFA

Passage 4 murine MSCs were grown on 4 well chambered slides and analysed for CD44 expression using IFA. CD44 was expressed in the adherent murine MSCs, with expression seen at cytoplasmic/cell membrane location. Negative controls confirmed absence of non-specific binding (Figure 6.3).

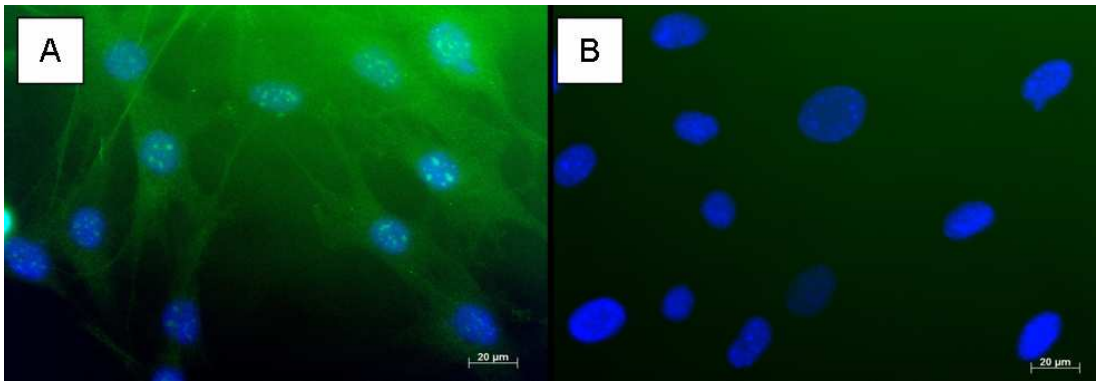


Figure 6.3. CD44 expression in murine MSCs. CD44 was found to be located predominantly in an extra-nuclear location, with some evidence of nuclear packaging (A). Negative controls were run alongside, labeled with secondary antibody only (B). DAPI was used for nuclear counterstain. Magnification x 400.

6.3.3 Cardiac Differentiation of Murine Mesenchymal Stem Cells

Cardiac differentiation was performed using the de-methylating agent 5'AZA over a three week time course as introduced in Chapter 5.

6.3.3.1 Morphology during Differentiation

Morphologically murine MSCs altered little during cardiac directed differentiation (Figure 6.4). No difference between cell populations in differentiated and undifferentiated wells were noted. Cell number increased rapidly, creating highly confluent wells from very early on during the experiment.

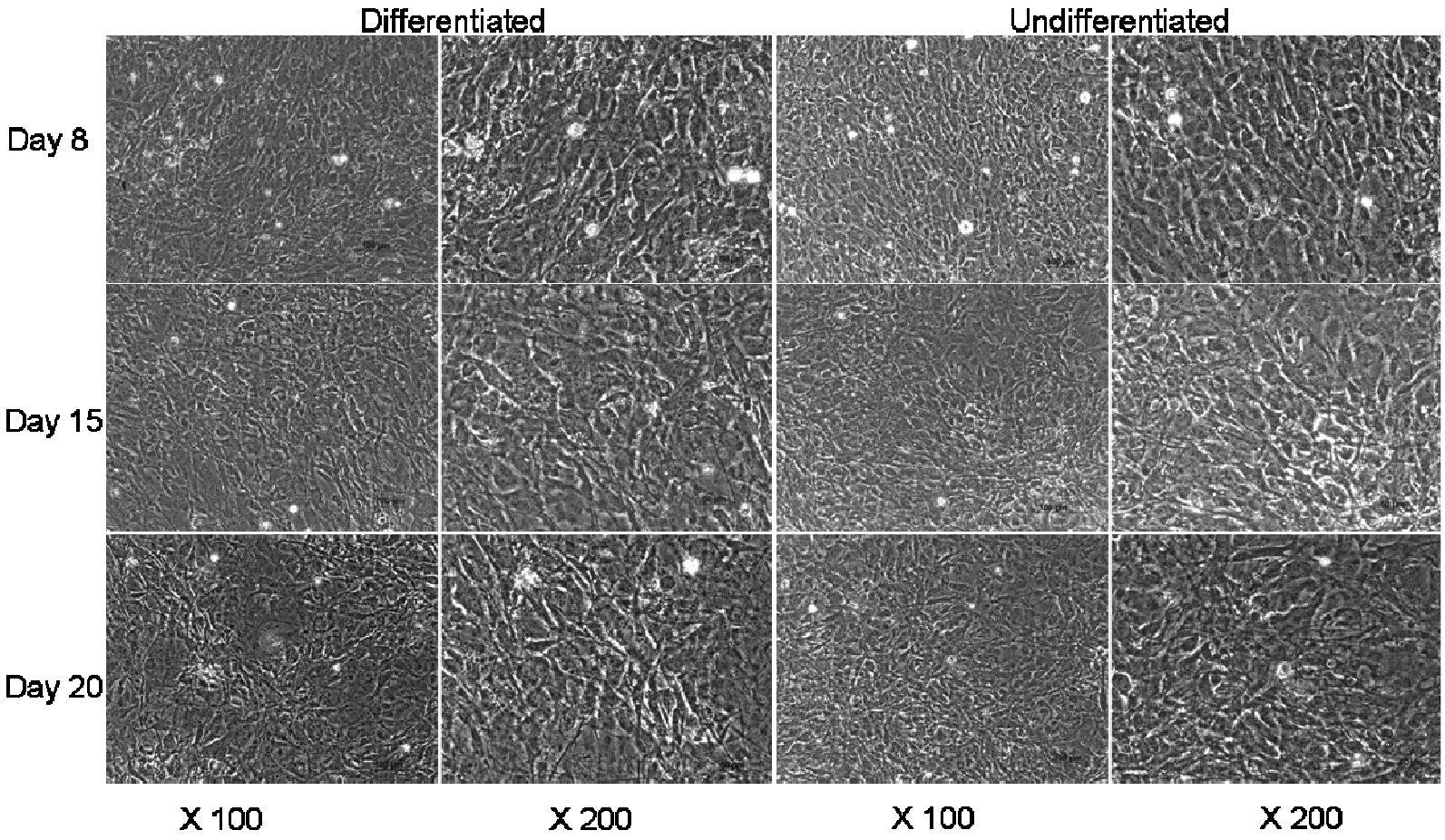

Differentiated
Undifferentiated
Day 8
Day 15
Day 20
X 100
X 200
X 100
X 200

Figure 6.4. Murine MSC differentiation toward cardiomyocyte lineages. Differentiated wells were compared to undifferentiated wells at three time points, day 8, 15 and 20 post initiation. Images were taken at magnification x 100 and x 200.

6.3.3.2 Semi-Quantitative RT-PCR Analysis of Cardiac Differentiation

Murine MSCs were analysed for marker expression using semi-quantitative RT-PCR and 100 ng of total RNA (Figure 6.5). Nkx2.5 and VEGF expression appeared to remain high and unchanged during cardiac directed differentiation in both cells undergoing differentiation and controls, compared to day 0 MSCs. GATA 4 appeared to increase in differentiated samples over time. Flk-1 appeared to have higher levels of expression in differentiated samples at all time points compared to day 0. SMA showed decreased expression in all samples when compared to day 0. CTI appeared to increase in all samples when compared to day 0. The ryanodine receptor was expressed at very low levels in all samples. CD44 was expressed in all samples, with higher level expression in differentiated cells compared to day 0. CD34 expression appeared to initially increase in differentiated cells at day 7, compared to undifferentiated, however this then shifted at day 14 and 21 with slightly higher expression seen in undifferentiated cells compared to differentiated. Connexin 43 appeared to be higher in all differentiated and undifferentiated samples when compared to day 0. The samples were negative for Islet 1, β_1-adrenergic receptor, c-Kit, vWF, CD45 and CTT at all time points. No difference was seen in expression patterns with murine MSCs cultured in standard (canine) MSC media.

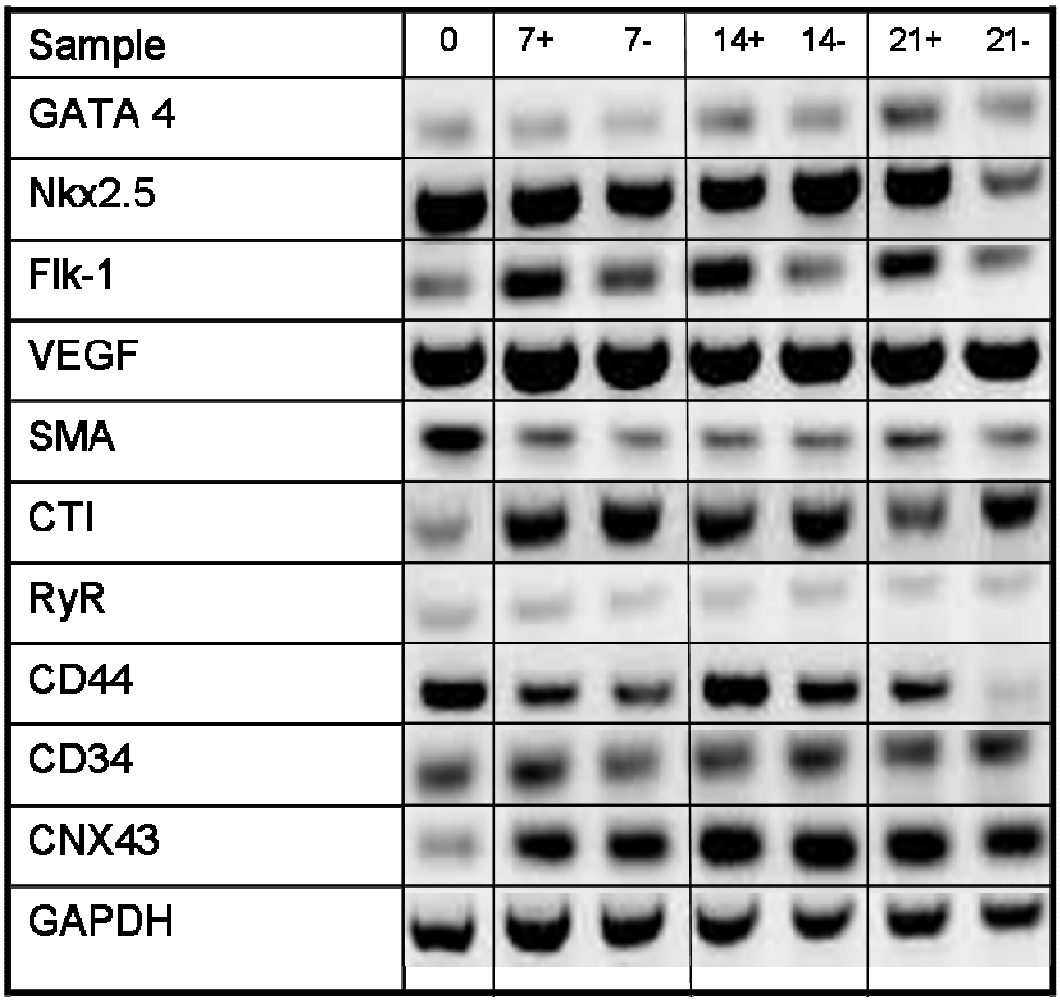


Figure 6.5. Semi-quantitative RT-PCR for marker expression of baseline MSCs (0) and cells undergoing differentiation (+) and controls (-) during cardiac directed differentiation over 7, 14 and 21 days. Vascular Endothelial Growth Factor, VEGF; Smooth Muscle Actin, SMA; Cardiac Troponin I, CTI; Cardiac Ryanodine Receptor, RyR; Connexin 43, CNX43.

6.3.4 Murine Marker Expression Changes with Passage

Murine MSCs were passaged for approximately 8 weeks, with 2 passages a week as described in Materials and Methods (section 6.2). Marker expression was compared using semi-quantitative RT-PCR between an early passage (passage 4; p(4)) and late passage (passage 18; p(18)) harvest of MSCs grown in both murine MSC and standard MSC media with 500 ng of total RNA input. Notable differences were seen in expression of several markers across passage number. CTT was found to be expressed in both passage 4 and 18 MSCs and an upregulation of Islet 1, GATA 4, CTT, CTI, CD44 and connexin 43 was seen, and a decrease in the expression of SMA (Figure 6.6). Cell numbers at passage remained consistent, with no evidence of senescence or morphological changes. No difference was seen between cells grown in murine MSC media compared to standard media.

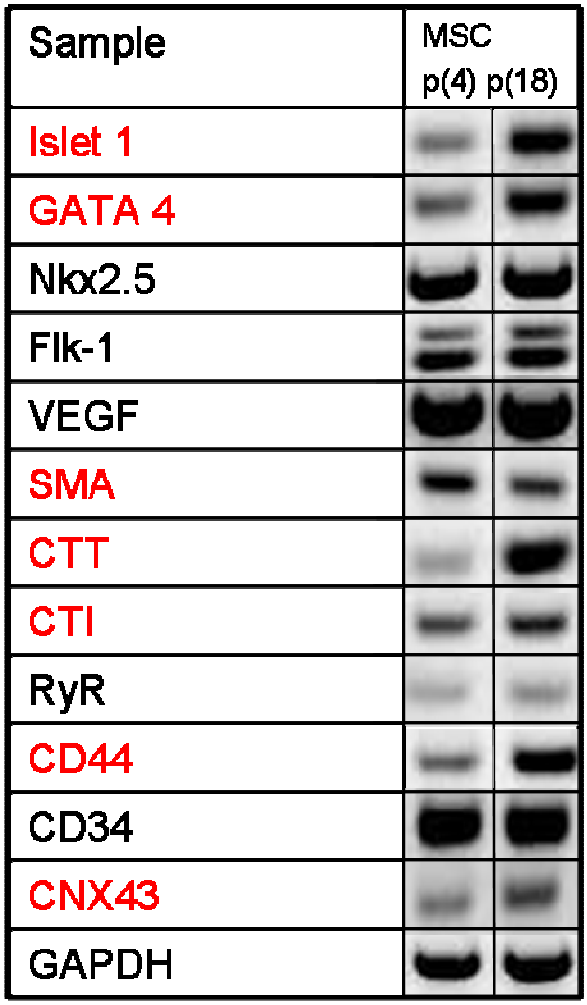


Figure 6.6. Murine MSC marker expression at early (P(4)) and late passage (P(18)). Differences were seen in expression of several markers (noted in red) with increasing passage number. Vascular Endothelial Growth Factor, VEGF; Smooth Muscle Actin, SMA; Cardiac Troponin T, CTT; Cardiac Troponin I, CTI; Cardiac Ryanodine Receptor, RyR; Connexin 43, CNX43.

6.4 Discussion

6.4.1 Murine Mesenchymal Stem Cell Culture and Characterisation

6.4.1.1 Isolation and Growth of Murine Mesenchymal Stem Cells

Initially murine MSCs were isolated in the same manner as for canine MSCs. However this technique failed to produce viable cells. This may be due to several factors. Firstly, when establishing murine MSC cultures it has been found that cellular plating density is crucial and a density which is either too high or too low prohibits the generation of long term cultured cells (Meirelles Lda and Nardi, 2003, Peister et al., 2004). This may have been influential in setting up our own primary murine MSCs, although our cell densities agreed most closely with those described by Meirelles *et al* they were significantly higher than those described by Peister *et al.* Another factor which may explain the unsuccessful primary culture was the technical difficulty in using Ficoll separation where the interface layer, which is usually apparent with canine MSCs, was not obvious. This layer is critical for collecting the appropriate cells and this established technique worked well with canine cells, but proved difficult with the mouse. We found at cell count that numbers appeared relatively high compared to those we obtained from canine bone marrow, however following the initial two days in culture a significant amount of free floating cells and debris were removed with the first wash. It is likely therefore that a large proportion of the original cells counted were not MSCs but in fact

haematopoietic and fat cells. Following the failed attempt to culture primary MSCs we then used a commercially available murine MSC cell line for all further experiments.

Murine MSCs obtained from a commercial source grew easily using the culture conditions described in the manual for the cells. These cells resembled other MSC populations described, and furthermore closely resembled the primary canine MSCs isolated during our experiments (Pittenger and Martin, 2004, Shim et al., 2004). The cells replicated rapidly, requiring regular passage once established. No evidence of senescence was seen during the culture of these cells and extended passage was achieved (passage 18) at which point experiments were ended. This may represent a more immortalized cell line than that found with primary canine MSCs and it is important to define this given the extent of comparisons made between murine models and primary cells from other species; both human and canine MSCs tend to senesce around passage 4-6, and this difference in culture behaviour may be relevant (Banfi et al., 2000, Sekiya et al., 2002).

6.4.1.2 Characterisation of Murine Mesenchymal Stem Cells

Characterisation of baseline MSCs found them to be CD44 positive by both IFA and RT-PCR, a crucial factor for defining an MSC population. Furthermore they were CD45 negative, ruling out haematopoietic contamination. Interestingly the murine MSCs were CD34 positive. Generally it is accepted that MSCs should be CD34 negative as mentioned in Chapter 5; Canine MSCs (Dominici et al., 2006). However, differences in

CD34 expression have been observed in murine MSCs, with interesting variation in the angiogenic potential between positive and negative populations (Copland et al., 2008). Furthermore CD34 expression is variable between strains of mouse used to isolate MSCs (Peister et al., 2004). Human MSCs have been described as both positive (Shim et al., 2004) and negative for CD34 (Pittenger et al., 1999, Etheridge et al., 2004, Xu et al., 2004, Antonitsis et al., 2007) and our isolated canine MSCs were predominantly CD34 negative (discussed below in 6.4.4). There is therefore a discrepancy in CD34 expression which needs to be taken into account when comparing different MSC populations, and the presence or absence of CD34 expression may define two separate MSC populations, each with their own differentiation ability, paracrine effects and angiogenic potential.

The purchased murine MSCs also expressed Flk-1 at a very low level which contradicts findings described by Peister *et al* and therefore reinforces variety within murine MSCs (Peister et al., 2004). Furthermore, our murine MSCs did not express c-Kit; c-Kit expression in murine MSCs is variable, but is generally shown to be negative (Baddoo et al., 2003). Meirelles *et al* described a bone marrow derived population of MSCs which were c-Kit low, and found that levels of c-Kit decreased with increasing passage, which may explain the lack of c-Kit expression in the purchased murine MSCs (da Silva Meirelles et al., 2006). Interestingly Orlic *et al* described a bone marrow derived stem cell population which was selected for c-Kit expression and was found capable of cardiac repair (Orlic et al., 2001a). More recently it has been found that a c-Kit enriched MSC population showed expression of cardiac markers (Grajales et al., 2010), therefore c-Kit expression may in fact be important for cardiac applications of MSCs. C-Kit is a

cell surface marker and could therefore be used for cell sorting, although as discussed in Chapter 4; Cardiac Stem Cells, cell sorting of canine cells for this marker was unreliable using MACS.

6.4.2 Cardiac Differentiation of Murine Mesenchymal Stem Cells

Using rodent models it has been suggested that MSCs are capable of cardiac differentiation *in vitro* using the de-methylating agent 5'AZA (Makino et al., 1999, Fukuda, 2001, Kruglyakov et al., 2006). We exposed early passage murine MSCs to cardiac differentiation based upon the technique described by Krugalykov *et al* and analysed the resultant cell populations at 3 weekly time points in differentiated and control cells compared back to day 0 baseline MSCs. We defined cardiac differentiation based upon behaviour in culture, including evidence of independent rhythmical beating and the expression of cardiac structural and functional genes. Using the culture behaviour and comprehensive panel of markers no conclusive evidence of cardiac differentiation was seen in the mouse MSC population following 5'AZA differentiation. However murine MSCs did show altered expression of several markers examined over the course of the differentiation experiment. GATA 4 appeared to increase in all samples over the time course, with a higher expression in the differentiated samples, and Flk-1 demonstrated higher expression in the differentiated samples at all time points when compared to the undifferentiated counterparts. CTI and CNXN 43 expression appeared higher in all samples when compared to day 0, whereas SMA appeared to decrease. No

beating was seen during the experiment and therefore it was deemed that cardiac differentiation did not occur.

6.4.3 Cardiac Gene Expression in Murine Mesenchymal Stem Cells

During the cardiac differentiation experiment, it was found that the murine MSCs expressed several key markers at baseline day 0 which are considered to be cardiac in origin, which had been previously described in mouse MSCs and implies a predetermined cardiogenic potential (Kruglyakov et al., 2006, Rose et al., 2008, Grajales et al., 2010). Murine MSCs expressed VEGF and Nkx2.5 at high levels and low levels of GATA 4, CTT, CTI, RyR and connexin 43 were also seen. Baseline MSCs did not express the β_1- adrenergic receptor. Taking the expression of these markers alone it could be argued that these cells have distinct cardiomyogenic potential, as already mentioned. However taking into account the apparent long term culture ability of these cells, it may also be that the mouse MSCs have a greater number of genes switched on, reflective of their more immortalized state.

6.4.3.1 Cardiac Gene Expression Alteration Over Time in Culture

Prompted by the suggestion that murine MSCs were altering gene expression pattern over time in culture we decided to investigate the effects of passage number on the murine MSCs by comparing early and late passage cells. No alteration in morphology was seen over the time period in culture but several markers demonstrated alterations in

expression level. Interestingly expression of two further cardiac markers; islet 1 and CTT was seen following an increase in total RNA input into the reverse transcription reaction (Figure 6.6), which suggested that RNA input is a critical factor when analyzing gene expression. We furthermore noted an increase in expression of islet 1, GATA 4, CTI, CTT, CD44 and connexin 43, and a decrease in the expression of SMA from early to late passage respectively. These results taken in isolation could suggest movement toward a cardiac lineage expression profile simply as a function of culture duration alone.

Key differences in expression of cardiac lineage genes in murine MSCs may be inadvertently used as proof of differentiation. Differentiation of MSCs *in vitro* is a contentious issue. Evaluating the literature, the criteria used to define differentiation is variable but generally based upon increases in cardiac lineage marker expression, cell morphology and cell behaviour in culture. However, when we take into consideration that the murine MSCs used in this study expressed genes deemed to be indicative of a cardiac phenotype at baseline and levels of expression altered by duration of culture it would be wrong to state successful differentiation driven by 5'AZA. Alteration in gene expression by passage number has been reported previously in murine MSCs (Meirelles Lda and Nardi, 2003). More recently Grajales *et* al described a c-Kit enriched bone marrow stem cell population which appeared to increase CTT expression due to time in culture alone, and suggested that increasing passage number improved the differentiation ability of bone marrow derived stem cells (Grajales et al., 2010). These results highlight the importance of interpretation of differentiation data, and in defining differentiation

criteria prior to the experiment particularly in the context of species type and passage number and illustrate the importance of appropriate controls.

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6.4.4 Comparisons Between Canine and Murine Mesenchymal Stem Cells

Canine and murine MSCs had significant similarity in morphology in culture. Furthermore, both cells could be passaged frequently (once-twice a week). However, canine MSCs senesced relatively early when compared to the continuous replication capacity of the murine MSCs seen during this experiment. Both canine and murine MSCs were positive for CD44. Canine MSCs were also positive for STRO-1 however this marker is not available for use in mouse cells. Both populations were negative for the haematopoietic marker CD45, reassuring that in both populations the cells had limited haematopoietic contamination. Both populations were also negative for c-Kit, a commonly used cardiac stem cell marker. Canine MSCs were predominantly negative for CD34, except for one population isolated (Chapter 5) whereas the mouse MSCs were consistently positive for CD34. Based on the literature already discussed there is heterogeneity in this marker expression, and this may reflect either different cell types or alternatively expression changes of this marker at different points in cell growth. Murine and canine MSCs expressed the following cardiac markers; Nkx2.5, Flk-1, islet 1, VEGF, SMA, and Connexin 43, but these markers in combination do not suggest a cardiac genotype. However murine MSCs also expressed GATA 4, CTT, CTI, and the ryanodine receptor which could be construed as cardiac in origin. Neither canine nor

murine MSCs expressed the β_1-adrenergic receptor, important in receiving adrenergic signaling in excitation of cells (Table 6.4)

Table 6.4. Marker expression of canine and murine MSCs

Marker	*Dog MSC*	*Mouse MSC*
CD44	+	+
CD34	+/-	+
CD45	-	-
c-Kit	-	-
Islet 1	+/-	+
Nkx2.5	+	+
GATA 4	-	+
Flk-1	+	+
CTT	-	+
CTI	-	+
B_1-Adrenergic Receptor	-	-
Cardiac Ryanodine Receptor	-	+
VEGF	+	+
vWF	-	-
SMA	+	+
Connexin 43	+	+

.

The differences seen between canine and murine MSCs may be explained by the original isolation technique employed. As shown here isolating murine MSCs using the traditional method did not work and furthermore this method has been found to generate a heterogeneous population of cells from the mouse including haematopoietic and fibroblastoid cells (Phinney et al., 1999). Therefore we purchased murine MSCs, and these cells were isolated using an alternative technique now commonly employed for murine cells which negatively selects against CD11b (Kopen et al., 1999, Baddoo et al., 2003, Tropel et al., 2004). The resultant mouse cell population will therefore not be a direct reflection of MSCs isolated in the traditional way and this may allow for the marker and culture characteristic difference described in this study.

6.4.5 Conclusion

This study highlights the differences seen in MSC populations. Species differences, site of MSC isolation, technique for isolation and passage number can all have an influence upon variation within MSCs. This study demonstrates the need for further work in characterising individual MSC populations including more global marker expression profiling studies and highlights the dangers in extrapolating successful differentiation based upon marker expression alone.

CHAPTER 7

Canine Mesenchymal Stem Cells and Cartilage Differentiation

Abstract

Mesenchymal stem cells (MSCs) have a capacity for multi-lineage differentiation thus providing a promising source of stem cells for regeneration. Due to the poor cardiac differentiation discussed in Chapter 5, it was decided to differentiate canine MSCs toward a connective tissue lineage, given that this is a key determinant of MSC characterisation, thus confirming that a true MSC population has been isolated. In this chapter primary canine MSCs isolated from the bone marrow were differentiated toward cartilage using standard 3D pellet culture and exposure to transforming growth factor β (TGFβ) and dexamethasone. The differentiated pellets were analysed for their expression of the chondrocyte marker SOX9, the cartilage markers collagen type I and II, and examined for the development of proteoglycan matrices indicative of functional cartilage. SOX9 was found expressed more widely in differentiated pellets compared to undifferentiated and collagen type II expression was seen to increase in differentiated pellets compared to undifferentiated with a decrease in collagen type I. Furthermore evidence of the development of proteoglycan matrix was seen in differentiated pellets.

This data suggests that successful formation of articular type cartilage from primary canine MSCs *in vitro* has occurred and that primary canine MSCs are therefore capable of differentiation.

7.1 Introduction

Cartilage disease and osteoarthritis (OA), a degenerative joint disease, are associated with high morbidity in pet dogs. Current treatment regimes rely on pain management, weight control and slowing of disease progression, however the development of new therapies is sorely required. MSCs have been postulated as a possible cell source for direct therapy. However, little baseline *in vitro* study of canine MSCs prior to their use therapeutically has been performed and therefore more research in this area is required. Furthermore, MSCs may offer the ability to study these progressive diseases *in vitro* allowing for the development of targeted novel therapeutics.

7.1.1 Mesenchymal Stem Cell Differentiation Toward Connective Tissue

By definition true MSCs must be capable of differentiation along multiple connective tissue routes (Dominici et al., 2006). The three routes of differentiation are predetermined by development factors; early in embryogenesis mesodermal cells develop to form mesenchyme, the precursor of all connective tissue, which has an inherent ability to migrate, a key factor required for normal embryonic development (Caplan, 1991). These mesodermal cells are the precursor to many structures within the body, and therefore MSCs have been isolated from several locations including bone marrow, adipose tissue and umbilical cord blood (Kern et al., 2006).

7.1.1.1 Osteogenic, Adipogenic and Chondrogenic *In Vitro* Differentiation

Studies across several species have shown that MSCs are readily capable of differentiating into osteoblast, adipocyte, and chondrocyte cell types *in vitro* (Gronthos et al., 1994, Yoo et al., 1998, Pittenger et al., 1999, Dennis et al., 2002, Suzdal'tseva et al., 2007). The techniques employed are standardized in the literature (Table 7.1);

Table 7.1. Techniques for MSC differentiation toward bone, fat and cartilage

Target	***Technique***
Osteoblast	Monolayer culture in DMEM media plus 10% FBS and dexamethasone, ascorbic acid and β-glycerophosphate
Adipocyte	Monolayer culture in DMEM plus 10% FBS and insulin, dexamethasone, isobutylmethylxanthine and indomethacin
Chondrocyte	Pellet (aggregate) culture in serum free DMEM in the presence of insulin-transferrin-selenium, sodium pyruvate, transforming growth factor β (TGFβ) and dexamethasone

Interestingly, initial site of MSC isolation can affect efficiency of differentiation: Umbilical cord derived MSCs are less able to differentiate into adipocytes (Kern et al., 2006) and bone marrow derived MSCs have superior potential for chondrogenic and osteogenic differentiation compared to adipose derived MSCs (Liu et al., 2007, Vidal et al., 2008).

7.1.1.2 Canine Mesenchymal Stem Cells and Connective Tissue Differentiation

Canine MSCs have been isolated from the bone marrow and differentiated toward osteogenic lineages successfully using both *in vitro* exposure to dexamethasone and also co-culture with primary autologous osteoblasts. Demonstration of an increased uptake of alkaline phosphatase and evidence of mineralisation implied formation of bone (Kadiyala et al., 1997, Csaki et al., 2009). Furthermore, canine MSCs have been isolated from umbilical cord blood and found capable of differentiation to all three connective lineages, as well as neuronal lineages reinforcing the multipotent differentiation potential of canine MSCs (Kamishina et al., 2006, Seo et al., 2009).

7.1.1.3 Pathways Controlling Mesenchymal Differentiation

It is believed that Wnt signalling closely controls the differentiation fate of MSCs (Etheridge et al., 2004, Ling et al., 2009). However more recently new pathways have been found which closely control the three differentiation routes of MSCs including the TGFβ, platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) signaling pathways (Ng et al., 2008). Using genome wide transcription analysis, Liu *et al* described the key regulators involved in differentiation of both bone marrow derived and adipose derived MSCs during differentiation along the three standard lineages. Interestingly, little difference was seen in marker expression between adipose and bone marrow derived MSCs during differentiation. Furthermore, a similar

early-differentiation pathway was seen for osteogenesis and adipogenesis, which was not seen for chondrogenesis. ZNF145 (a promyelotic zinc finger protein) and carboxypeptidase M (a cell membrane metalloprotease) were both upregulated in the osteogenic and adipogenic pathways whereas dermatopontin (an extracellular matrix protein) and collagen 10A1 were upregulated in chondrogenic pathway. By late stage differentiation, each pathway had its own separate set of signaling molecules, allowing for terminal differentiation; for osteogenesis Osteomodulin (OMD), Apolipoprotein D (APOD) and ZNF145; for adipogenesis Adiponectin (ACDC), Fatty Acid Binding Protein 5 (FABP5) and Low Density Lipoprotein (LDL). For cartilage differentiation, a further increase in the expression of the early stage markers at late differentiation triggered terminal differentiation suggesting the importance of this pair of markers in the formation of cartilage from mesenchymal tissues (Figure 7.1) (Liu et al., 2007).

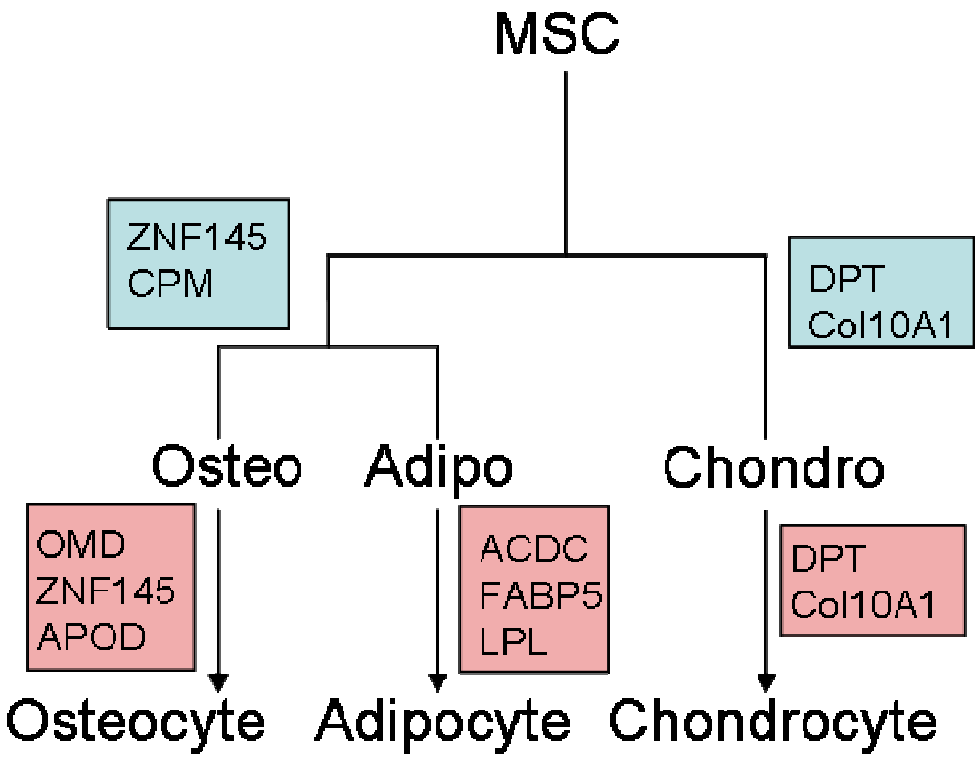


Figure 7.1. Differentiation pathways for MSCs from both bone marrow and adipose sources along connective tissue routes. A close relationship between the control of differentiation of osteocytes and adipocytes is seen at early stages whereas chondrocytes breakaway early in differentiation and follow their own path (factors in blue). At late stage differentiation each cell type is influenced by its own signaling factors (factors in pink). Carboxypeptidase M (CPM), Collagen 10A1 (Col10A1), Dermatopontin (DPT) Osteomodulin (OMD), Apolipoprotein D (APOD), Zinc Finger Protein 145 (ZNF145), Adiponectin (ACDC), Fatty Acid Binding Protein 5 (FABP5), Low Density Lipoprotein (LDL). Based upon Liu *et al* (Liu et al., 2007).

7.1.2 Articular Cartilage Defects and the Role of Cellular Therapy

Articular cartilage is a tough connective tissue which protects articular surfaces, acts a shock absorber and participates in the fluid biomechanics of the joints associated with it. Damage to articular cartilage has long been known to be a precursor to the development of OA (Cox and Cordell, 1977, Ghosh et al., 1990, Berjon et al., 1991, Little et al., 1996, Lindhorst et al., 2000). Management of OA is based upon pain control and slowing of disease progression, however ultimately the condition always advances over time. This has led to the investigation of novel treatments and the use of cells as a source of therapy.

7.1.2.1 Chondrocytes in Cartilage Repair

Chondrocytes are the cells which are found within cartilage and secrete collagen and extracellular matrices. A loss of functional chondrocytes leads to the degeneration of cartilage and is a forerunner to the development of OA. Chondrocytes have been investigated as a direct source of treatment for damaged cartilage with some evidence of success in animal models (Ibarra et al., 1997, Peretti et al., 2004). Autologous chondrocytes have been used as treatment options in human knee joint disease since 1987 and were found to improve damaged tissue. Research into improving this as a treatment option still continues (Brittberg et al., 1994, Brittberg et al., 2001).

Use of chondrocytes for therapeutics is limited by the difficulty in isolating the cells in significant numbers. Furthermore, it has been shown that cultured articular chondrocytes de-differentiate in long term monolayer culture toward fibrochondrocytes, the cells which contribute to the formation of scar cartilage. This alters both expression of collagen class from II toward I and the balance of the proteoglycan matrix, which has major ramifications in the scale up of these cells in culture prior to their use therapeutically (Benya et al., 1978, Stokes et al., 2002).

7.1.2.2 Mesenchymal Stem Cells in Cartilage Repair

Using animal models it has been suggested that MSCs may offer an alternative cellular therapy to chondrocytes when injected into damaged cartilage. However, based on current research improvement in healing is seen at short time points, and with as yet no evidence of long term repair (Wakitani et al., 1994, Wilke et al., 2007). Black *et al* from Vet-Stem, California, performed two studies injecting adipose derived MSCs directly into the coxofemoral or elbow joints of dogs suffering from chronic osteoarthritis and found that in both diseases a significant improvement was seen in lameness, range of motion and pain on manipulation over time, when compared with baseline values (Black et al., 2007, Black et al., 2008). However little characterization was performed prior to the implantation of these MSCs, and due to the isolation technique a mixed population of cells was likely to have been injected, therefore although these results are promising, more basic science needs to be performed to support this data.

It is not yet known how MSCs exert their effect when used therapeutically; they may act via a paracrine signaling mechanism or direct cell replacement, or a combination of both. This is currently a highly researched field, but it can be seen from the literature that there is commonly an improvement when MSCs are used. MSCs can be used in their primary form, or pre-differentiated into chondrocytes before implantation. Comparison between injection of MSCs versus MSC derived chondrocytes demonstrated an improved repair in MSC treated populations which was not seen in the MSC derived chondrocyte treated populations. This may indicate that prior differentiation of the MSC population limits tissue incorporation or alternatively a paracrine effect induced by released factors from MSCs may be responsible which is lost upon terminal differentiation of the cells (Meirelles Lda et al., 2009, Pabbruwe et al., 2010). Several clinical trials are now underway investigating the use of autologous MSCs for specific joint diseases in humans (Clinical Trials.gov) and the results of these trials are awaited with great expectation.

7.1.2.3 Cell Laced Scaffolds for Cartilage Repair

Therapeutics using both chondrocytes and MSCs often use a scaffold as a method for inserting the cells into damaged tissue. Non-synthetic allogenic scaffolds were commonly used early in research. Peretti *et al* used devitalized allogenic meniscal cartilage to lace chondrocytes onto prior to implantation in pigs which showed bonding of the implant, and improved healing in treated joints versus controls (Peretti et al., 2004). Synthetic scaffolds are now widely available and a huge variety are accessible in

the field (scaffolds reviewed in (Stella et al., 2010)). Efficiency of chondrocyte differentiation using MSCs within hydrogel or gelatin scaffolds shows no difference between substance used, however biomechanical properties of the subsequent cartilage can vary significantly (Awad et al., 2004). Furthermore, it has been seen that type of scaffold used is significant in the migration and differentiation of MSCs in the *in vivo* environment (Pabbruwe et al., 2010). The use of scaffolds for MSC implantation is an exciting developing area of research.

7.1.3 Aims

In this Chapter we will take previously characterized canine MSCs (first discussed in Chapter 5) and differentiate them towards cartilage using traditional techniques. The resultant cell population will be analysed using multiple techniques, and in particular for evidence of successful articular type cartilage differentiation.

7.2 Materials and Methods

7.2.1 Isolation and Characterisation of Canine Mesenchymal Stem Cells, Chondrocytes and Fibroblasts

7.2.1.1 Isolation of Canine Mesenchymal Stem Cells

Canine MSCs were isolated from bone marrow and cultured and characterised as described previously (see Chapter 5; Canine Mesenchymal Stem Cells and the Cardiovascular System).

7.2.1.2 Isolation of Canine Chondrocytes

Articular cartilage from the femoral head of two recently euthanized dogs was dissected into shavings using a scalpel and placed into 10 mL DMEM media with 10% FBS and 100 U/mL penicillin G and 100 μg/mL streptomycin (all Invitrogen, UK) and chilled on ice. These were then centrifuged at 1500 rpm for 5 minutes at room temperature to move shavings to the base of the Falcon tube. Medium was removed and a 0.2% trypsin/0.4% collagenase IV (Invitrogen, UK) digestion solution was added onto the shavings and incubated at 37 °C for 30 minutes in a water bath. The shavings were then centrifuged at 1500 rpm for 5 minutes at room temperature and the original trypsin/collagenase removed and fresh added. The shavings were then incubated again at 37 °C for a further 30 minutes, before a repeat centrifuge and trypsin/collagenase removed. Fresh

trypsin/collagenase was added and the shavings incubated for 180 minutes at 37 °C. The shavings were then centrifuged a final time and the trypsin/collagenase discarded and the shavings washed in chondrocyte media; DMEM Hams F12 (Invitrogen, UK), with 10% FBS and 100 U/mL penicillin G and 100 μg/mL streptomycin. A final centrifuge was performed and media discarded and the shavings resuspended in 5 mL of chondrocyte media. This was then passed through a 100 μm diameter filter membrane to release cells and the flow through collected. The filter was washed through with a further 5 mL of chondrocyte media, and the flow through added to the suspension. The cell suspension was then centrifuged at 1500 rpm for 5 minutes to pellet the cells. Cells were resuspended in 1 mL of chondrocyte media and counted as previously described. A cell count of 1.5 x 10^5, and 2.2 x 10^6 was achieved for the first and second dog respectively and each was placed into a fibronectin coated T25 at 37 °C, 5% CO_2. A partial media change was performed after 5 days, and at 7 days culture flasks for both dogs had become confluent and were passaged into T75 tissue culture flasks with media changes every 3-4 days.

7.2.1.3 Isolation of Canine Fibroblasts

1 cm sections of clipped full thickness skin was harvested from the lateral thoracic region from two recently euthanized dogs (as for chondrocytes) and placed into 10 mL chondrocyte media; DMEM low glucose media with 10% FBS and 100 U/mL penicillin G and 100μg/mL streptomycin and chilled on ice. Skin sections were then transferred into a digestion solution of 0.2% trypsin/ 0.4% collagenase and incubated at 37 °C for

30 minutes in a water bath. Sections were manually removed from the digestion solution using forceps and placed into PBS to wash the sections. Two washes were performed and hair was scraped away from the skin before a final PBS wash. The skin was then cut into small sections and placed into 7 mL chondrocyte media and transferred to fibronectin coated T25 flasks and incubated at 37 °C, 5% CO_2. Following 4 days in culture 4 mL of chondrocyte medium was removed to allow skin sections to contact the base of the flask to stimulate attachment, and 48 hours later 4 mL of fresh chondrocyte medium was replaced. At 1 week post harvest skin sections were removed from T25 culture flasks and discarded and 6 mL medium was removed and replaced with fresh chondrocyte medium.

7.2.2 Mesenchymal Stem Cell Differentiation Toward Cartilage

Two techniques were employed to differentiate canine MSCs to cartilage.

7.2.2.1 Cartilage Differentiation using Matrigel™

Matrigel was purchased from BD Biosciences, UK and defrosted at 4 °C on ice overnight. Using pre-chilled pipettes 5 mL of matrigel were transferred to 1.5 mL of DMEM low glucose media, to make a total of 6.5 mL. Canine mesenchymal stem cells (passage 1), chondrocytes (passage 2) and dermal fibroblasts (passage 1) were harvested from their respective flasks and counted as previously described. 1 x 10^5 cells/mL of each cell type were added to the matrigel solution and kept chilled. A plate plan using a

48-well tissue culture plate (Costar, UK) was set up as below (Figure 7.2) including wells for each cell type, with differentiated and undifferentiated MSCs, and 3 time points; 7, 14 and 21 days in triplicate. 175 μL of appropriate matrigel cell suspension (1.75×10^4 cells total) was added to the base of each appropriate well on the 48-well plate. The plate was then placed at 37 °C for 30 minutes to allow matrigel to set. Following this, 300 μL of appropriate media was added to each well. For MSC differentiation media consisted of; DMEM high glucose (Invitrogen), 1% ITS+ premix (BD Biosciences, UK), 10^{-7} M Dexamethasone (Autogen Bioclear, UK), 1 mmol/L Na Pyruvate (Gibco, UK), 10 ng/mL TGFβ1 (Peprotech) and 100 U/mL penicillin G and 100 μg/mL streptomycin (both Invitrogen). For MSC undifferentiated wells standard MSC media was added consisting of; DMEM low glucose containing Glutamax-I (Invitrogen), with 10% FBS (Invitrogen) and 100 U/mL penicillin G and 100 μg/mL streptomycin (both Invitrogen). For both chondrocyte and fibroblast wells chondrocyte media was added. The plate was then placed at 37 °C, 5% CO_2. A media change for each well was performed at 3 days, and first harvest of cells collected at day 7. Day 0 pellets of each cell type were snap frozen and stored at -80 °C for later use.

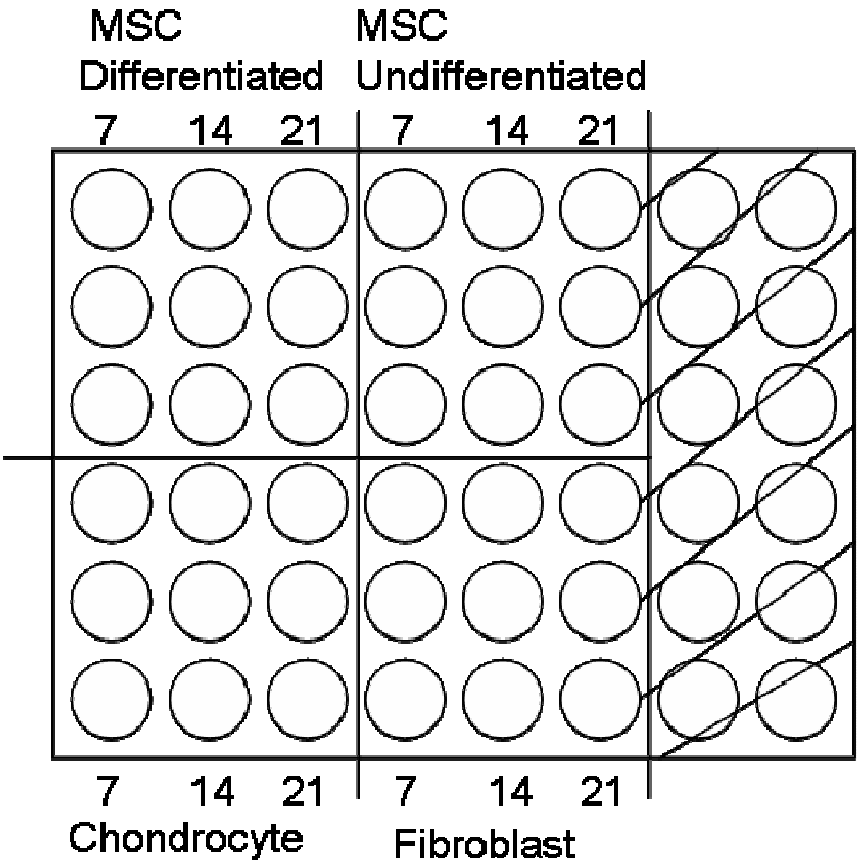


Figure 7.2. 48-well plate layout for MSC differentiation to chondrocyte using matrigel. Three cell types were used; MSCs, chondrocytes and fibroblasts from the same dog. Wells were run in triplicate. Harvests of cells were collected at days 7, 14 and 21.

7.2.2.1.1 Harvest of Cells from Matrigel™

Cells were harvested at each time point from matrigel using Matrigel Cell Recovery Solution (BD Biosciences, UK). Media was removed and discarded and the cells washed three times with PBS. 700 μL of recovery solution was added to the surface of the matrigel and the cell/gel layer was scraped into an ice cold 50 mL Falcon tube. The well

was rinsed with a further 2 mL of cell recovery solution which was added to the Falcon tube. The tube was inverted several times and left on ice for 1 hour to dissolve matrigel completely. The cell suspension was then centrifuged at 300g for 5 minutes at 4 °C, and the resultant supernatant discarded. The cell pellet was washed with chilled PBS, and re-centrifuged. A second PBS wash was performed and the triplicate pellets pooled before a final centrifuge. Following this the PBS was removed and Falcon tube examined for the presence or absence of a cell pellet.

7.2.2.2 Cartilage Differentiation using 3D Pellet Culture

2 x 10^5 canine MSCs were placed into either standard MSC media or cartilage differentiation media in 15 mL Falcon tubes. Cells were spun at 500g for 2 minutes, and placed at 37 °C, 5 % CO_2 overnight. After 12 hours small pellets formed in the base of the Falcon tubes which were free floating in the media. These pellets were maintained over the period of the experiment, with media changes performed every 2-3 days with either standard MSC media or cartilage differentiation media. Pellets were harvested at days 7, 14 and 21 for RNA extraction and histopathology and immunohistochemistry, with each experiment run in duplicate. Day 0 MSCs were snap frozen and stored at -80 °C at initial set up as a baseline control. Extracted RNA was used for quantitative RT-PCR analysis using 50 ng of extracted RNA and primers for collagen I and collagen II.

A repeat experiment was performed using an initial higher cell number input to create the 3D pellet. 2 x 10^6 MSCs were added to the Falcon tube prior to initial centrifuge in 1 mL of appropriate media, followed by an exact copy of the original experiment. RNA was extracted from the final pellets and used for downstream quantitative RT-PCR reactions.

7.2.2.2.1 Immunohistochemistry of Differentiated Pellets

Cellular pellets were transferred from media to HistoGel (Thermo Scientific, UK) to fix prior to paraffin wax embedding, and then sectioned using microtome cutting. Immunohistochemistry staining was performed by Neil Macintyre, Laboratory Manager, Histology and Cytology, R(D)SVS, University of Edinburgh.

7.2.2.2.1.1 Collagen Type II

The sections were dewaxed using xylene (Table 7.2) and a stepwise protocol described in Table 7.3;

Table 7.2. Dewaxing stages for paraffin embedded sections.

Xylene (De Wax solutions)	***15 minutes***
100% ethanol	10 minutes
95% ethanol	10 minutes
90% ethanol	10 minutes
70% ethanol	10 minutes
water	10 minutes

Antigen retrieval was performed using enzyme treatment; 2 mg/mL hyaluronidase (Sigma) with 0.125 U/mL chondroitinase (Sigma) in Tris-buffered saline, pH 7.6. The enzyme solution was warmed at 37°C along with the sections for 30 minutes prior to enzyme treatment for 2 hours at 37 °C. Sections were subsequently placed into distilled water and then placed into Thermo Fisher Scientific Sequenza coverplates in TBST (50 mM Tris.HCl, 150 mM NaCl, 0.1% Tween 20) buffer pH 7.6. Collagen II rabbit polyclonal antibody (Abcam, UK. Catalogue number Ab34712) was diluted 1/50 using Dako antibody diluent (Dako, UK. Catalogue number S0809) and 100 µL applied to the slides and placed at 4 ^{o}C for 16 hours. The sections were then placed at 25 °C in an incubator for 30 minutes (and all reagents warmed to 25 °C for 30 minutes). The sections were then rinsed in TBST, pH 7.6 and then loaded onto the Thermo Fisher Scientific Autostainer 360 and rinsed again in TBST, pH 7.6, before staining using the UV Quanto protocol;

Table 7.3. UV Quanto protocol for immunohistochemistry

Step	Time
Non specific peroxidase block (Dako, UK REAL blocking agent. Catalogue number S202386)	10 minutes
TBST rinse	
Antibody amplifier (Thermo Fisher Scientific, UK. Catalogue number TL-125-QHL)	10 minutes
TBST rinse	
HRP Polymer (Thermo Fisher Scientific, UK. Catalogue number TL-125-QHL)	10 minutes
TBST rinse	
DAB buffer substrate (Newmarket Scientific, UK. Monosan Dab substrate kit. Catalogue number. MON-APP177).	2 minutes
TBST rinse	
DAB buffer substrate	10 minutes
TBST rinse	
Distilled water rinse	
DAB Enhancer (Newmarket Scientific, UK. DAB concentrate. Catalogue number CO7-25).	3 minutes
TBST rinse	
Counterstain in Harris haematoxylin	30 seconds
Wash in tap water	1 minute
Blue up using Scott's Tap water substitute	2 minutes
Dehydrate and clear through ethanol and xylene (as above)	2 minutes each
Mount in ClearVue mountant (Thermo Fisher Scientific, UK)	

Positive controls were performed using canine articular cartilage. Negative controls were performed using canine articular cartilage and antibody diluent only.

7.2.2.2.1.2 SOX9

The sections were dewaxed as previously described. SOX9 antigen retrieval was performed using Heat Induced Epitope Retrieval (HIER), using Histos 5 microwave antigen retrieval system (Leica Microsytems, Germany). Sections were placed at 110 °C for 15 minutes in 0.01 mol/L citric acid, pH 6.0. Sections were allowed to cool then placed into distilled water. Sections were then placed into Thermo Fisher Scientific Sequenza coverplates in TBST. SOX9 rabbit polyclonal (Abcam, UK. Catalogue number Ab3697) was diluted 1:50 with Dako antibody diluent and then added to the sections. The sections were then incubated at 25°C for 1 hour, with subsequent staining using the UltraVision Quanto Detection System (Thermo Fisher Scientific, UK) as above. Positive controls were performed using canine articular cartilage. Negative controls were performed using canine articular cartilage and antibody diluent only.

7.2.2.2.2 Toluidine Blue Staining of Differentiated Pellets

Cartilage sections were rinsed in distilled water, and then placed into 1% aqueous Toluidine Blue (Sigma, UK) for 30 minutes at room temperature. The sections were then rinsed in distilled water and de-colourised using acid alcohol (1% HCL in 70% ethanol), diluted 1:4 for 5 seconds. The slides were then rinsed in distilled water again, before air

drying and clearing in Xylene (as above). The slides were then mounted in DPX mounting media (VWR, UK). Toluidine blue staining of slides was performed by Neil Macintyre (as above).

7.2.2.2.3 Quantitative RT-PCR Analysis of Cartilage Differentiation

Primer and probe designs for collagen type I and II were generously donated by Dr Dylan Clements. Sequence details were aare described in Table 7.4;

Table 7.4. Primer sequences and product information for collagen type I (Col1A2b) and II (Col2A1).

Target	*Forward 5'-3'*	*Tm (°C)*	*Reverse 5'-3'*	*Tm (°C)*	*Size (BP)*	*Probe (Roche,UK)*
Col1A2b	CTA TCA ATG GTG GTA CCC AGT TT	59	TGT TTT GAG AGG CAT GGT TG	59	111	40
Col2A1	CTG GTG AAC CTG GAC GAG AG	60	ACC ACG ATC ACC CTT GAC TC	59	86	53

Primers and probes were ordered and prepared as described previously in Chapter 2; Materials and Methods.

7.2.2.2.3.1 cDNA synthesis

RNA extracted from cartilage differentiation pellets was reverse transcribed as described in Chapter 2; Materials and Methods.

7.2.2.2.3.2 PCR Master mix

A master mix was prepared for the target genes and 18s rRNA are described in Tables 7.5 and 7.6 below;

Table 7.5. Target gene master mix for collagen type I and II

Master mix	***1x (µL) (n x =number of wells)***
Realtime Master mix	5
10x Primer/Probe (FAM)	0.3

A master mix was also prepared for the 18s rRNA housekeeping control gene:

Table 7.6. 18s rRNA control gene master mix

Master mix	***1x (µL) (n x =number of wells)***
Realtime Master mix	5
20x 18s VIC Control	0.15
Water	0.15

7.2.2.2.3.3 Primer Efficiency and Optimisation

Real time plates and cover foils were ordered from Roche, UK. RNA was extracted from canine articular cartilage and 50 ng of total RNA was used for reverse transcription. Neat cDNA was serially diluted 1:5, 1:10, 1:50 and 1:100 and a plate plan was devised for primer pair optimisation (Figure 7.3).

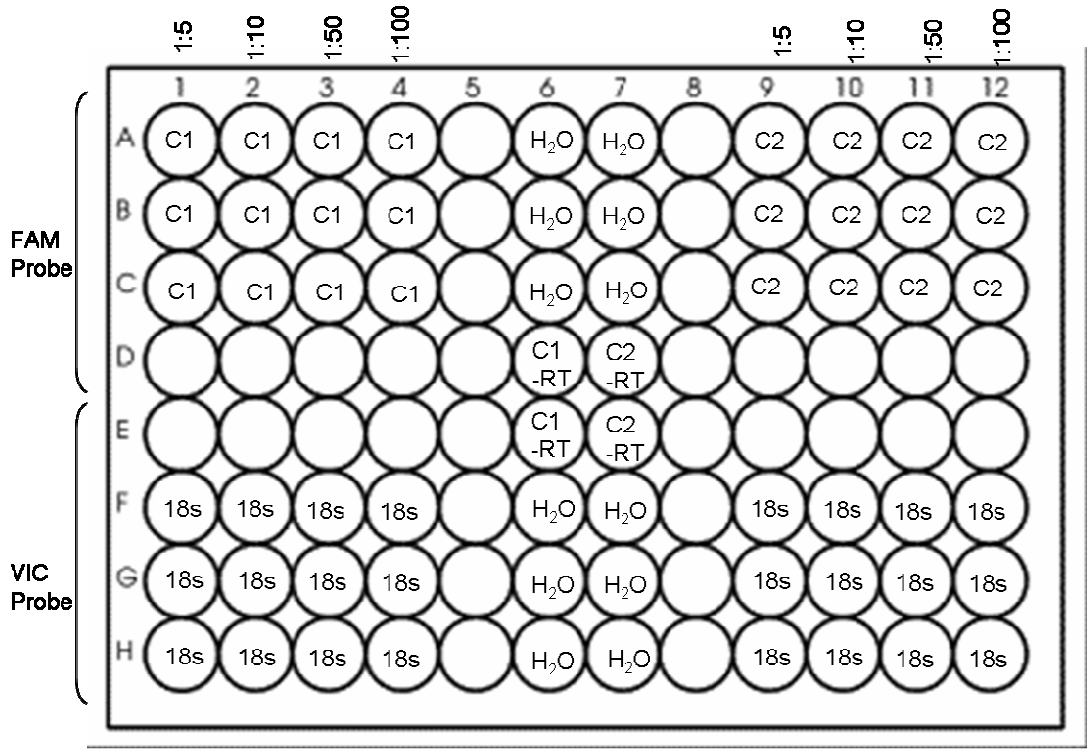


Figure 7.3. 96-well plate plan for collagen type I and II primer optimization. Wells were designed to allow for triplicates of articular cartilage cDNA dilutions (1:5, 1:10, 1:50, 1:100) run with primers for collagen type I (C1) and collage type II (C2). Triplicate wells of water negative controls were run for all primers, and –RT wells of articular

cartilage RNA were run with each primer. Primers for collagen type I and II were bound to a FAM probe, and primers for 18s rRNA in-house gene positive control bound to a VIC probe.

5.3 µL of appropriate master mix (FAM - collagen I and II; VIC - 18s rRNA) and 4.7 µL of appropriate cDNA dilution was added to each well. 4.7 µL of diluted sample RNA (-RT) or DEPC treated water was added to each control well. Wells were sealed shut using a foil and centrifuged at 1500g for 2 minutes to pool samples into base of wells. Plates were placed into the Roche Lightcycler 480 and run on a relative analysis programme (Table 7.7):

Table 7.7. Programme data for Roche Lightcycler 480

Programme	***Cycle***	***Acquisition***
Pre-incubation	1	N
Amplification	50	Quantify
Cooling	1	N

With individual cycles as described in Table 7.8:

Table 7.8. Temperature settings per cycle for Roche Lightcycler 480

	Temperature °C	*Acquisition*	*Hold (Time)*	*Ramp*
Pre-Incubation	95	N	10 minutes	4.4
Amplification (50x)	95	N	10 seconds	4.4
	54	N	30 seconds	2.2
	72	Single	1 second	4.4
Cooling	40	N	10 seconds	1.5

Data was exported to Microsoft® Excel and a primer efficiency curve was generated including an R^2 value, which gave an indication of efficiency of reaction.

7.2.2.2.3.4 Relative Gene Expression Analysis

Following primer optimisation samples could be compared for relative expression of specific gene targets. A plate plan for relative expression of collagen I and II is shown below (Figure 7.4):

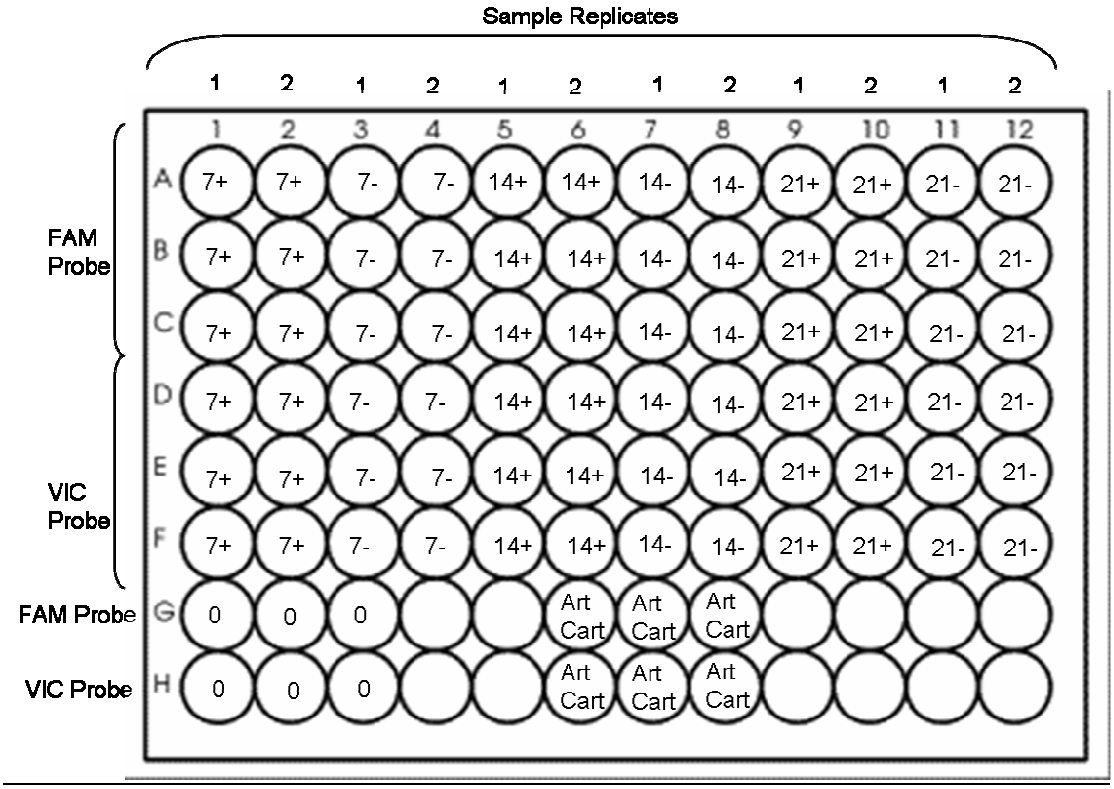


Figure 7.4. 96-well plate plan for collagen type I and II gene expression analysis. Wells were designed to allow for triplicate technical replicates of sample cDNA dilutions with sample replicates run separately (1,2) for each time point (7, 14 and 21 days) for both differentiated (+) and undifferentiated (-) cells. Triplicate wells of Day 0 cDNA were run alongside with articular cartilage cDNA run as positive control. Primers for collagen type I and II were bound to a FAM probe, and primers for 18s rRNA positive control bound to a VIC probe.

This plate design allows for comparison between day 0, 7, 14 and 21 differentiated and undifferentiated samples. Articular cartilage cDNA was added to allow for a reference control sample.

All negative controls were run on a separate plate, due to plate space limitation, using the same master mix that was used for the sample plate thereby eliminating master mix error (Figure 7.5).

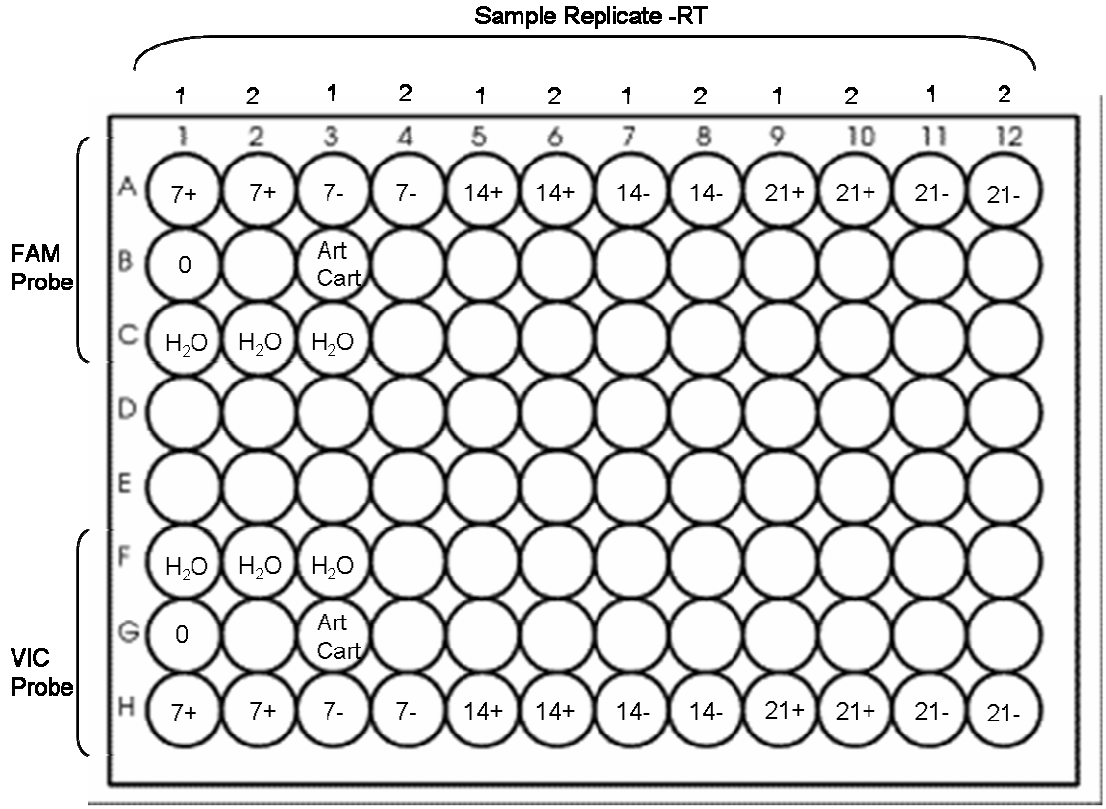


Figure 7.5. Plate plan for negative control samples from MSC differentiation to cartilage. Water negative controls were run in triplicate, and –RT wells of each sample duplicate and articular cartilage control were run with each primer using the same master mix as for relative expression analysis.

7.3 Results

7.3.1 Canine Primary Cell Culture

7.3.1.1 Canine Mesenchymal Stem Cell Behaviour in Culture

Canine MSC behaviour in culture is discussed in Chapter 5; Canine Mesenchymal Stem Cells and the Cardiovascular System

7.3.1.2 Canine Chondrocyte Behaviour in Culture

Canine chondrocytes adhered to fibronectin coated tissue culture flasks readily, and cell replication was rapid. An adherent monolayer of cells morphologically similar to fibroblasts cells grew rapidly, and required passage by 1 week post harvest (Figure 7.6).

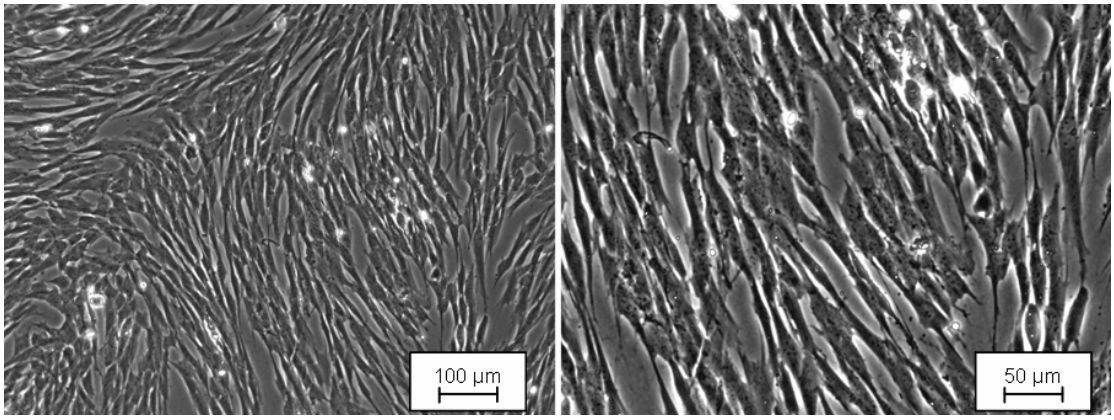


Figure 7.6. Canine chondrocytes in culture. Morphologically chondrocytes assumed a fibroblast-type morphology. Magnification x 100 (left) and x 200 (right).

At passage the trypsinised cells assumed a spherical morphology, which subjectively appeared green in colour. Following cell removal from the flask obvious cell secretions were present on the bare tissue culture plastic surface, suggestive of cellular secretion (Figure 7.7). Chondrocytes were readily passaged and adhered to new tissue culture flasks within 24 hours.

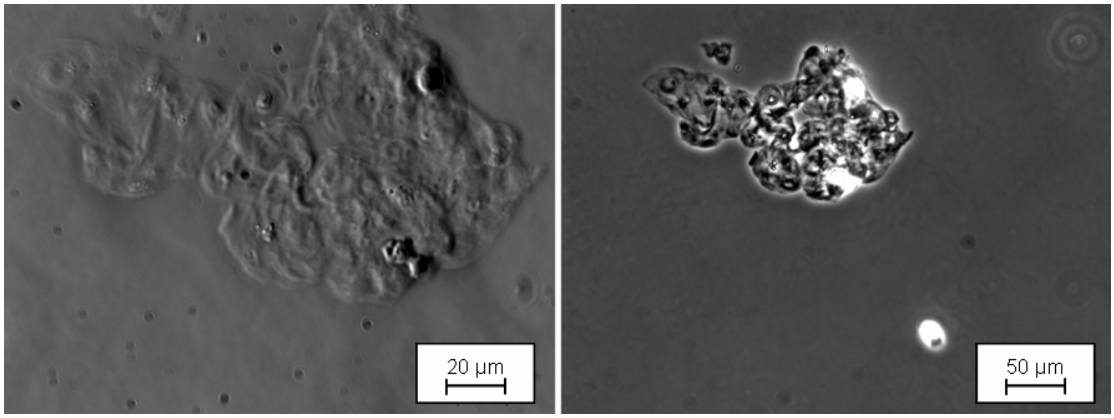


Figure 7.7. Chondrocyte secretions. Following passage adherent secretions could be seen attached to the base of the flask. Magnification x 400 (left) and x 200 (right).

7.3.1.3 Canine Fibroblast Behaviour in Culture

Canine dermal fibroblasts grew out from skin explants in small patches. Explants were removed from culture after 1 week, following which dramatic cell replication occurred. The majority of the cells appeared morphologically like fibroblasts, with adherent, flattened elongated cells which were distinct from canine chondrocytes (Figure 7.8).

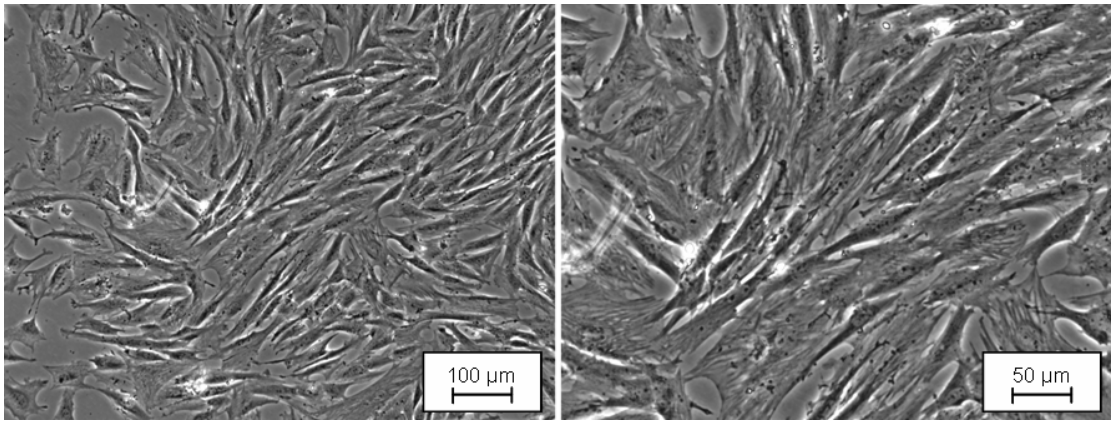


Figure 7.8 Canine dermal fibroblasts in culture. Morphologically dermal fibroblasts were adherent spindle shaped cells with long projections contacting other cells. Magnification x 100 (left) and x 200 (right).

However there was some heterogeneity in cell morphology, with some spheroid cells suggestive of adipocytes (Figure 7.9)

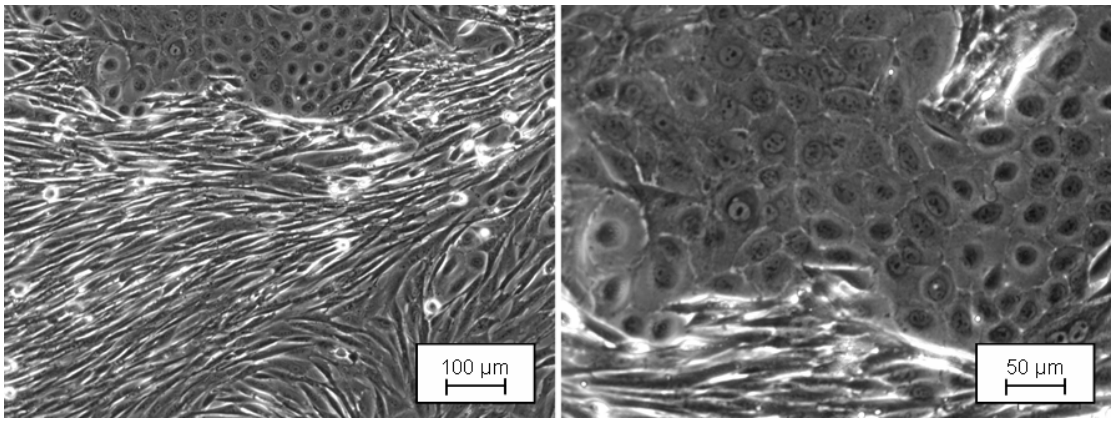


Figure 7.9. Mixed population of cells from skin explant culture. Following skin explant culture a mixed population of cells was present, with spherical globular cells similar to adipocytes surrounded by fibroblast like cells. Magnification x 100 (left) and x 200 (right).

7.3.2 Canine Mesenchymal Stem Cell to Cartilage Differentiation

7.3.2.1 Cartilage Differentiation using Matrigel

A differentiation experiment for MSCs was devised using the semi-solid growth substrate matrigel in differentiation media. Control wells were run using MSCs in standard MSC media, and chondrocytes as a positive control and dermal fibroblasts as a negative control. All three cell types were harvested from two dogs, however only cells from one dog became ready for experimentation at the same time. Cells were mixed into matrigel and plated up as described in materials and methods above. 24 hours after plating cells could be seen within the matrigel for all cell types and by 3 days post set-up MSCs, chondrocytes and fibroblasts all looked morphologically similar. At day 7 the first harvest of cells was performed using matrigel cell recovery solution. Following harvest no cell pellets could be seen in individual Falcon tubes, and therefore triplicates were pooled before a final centrifuge. However no cell pellet was present again, and therefore Falcon tubes discarded. Before day 14 harvest there was fungal contamination within the wells of the 48 well plate. The experiment was therefore abandoned due to technical difficulty.

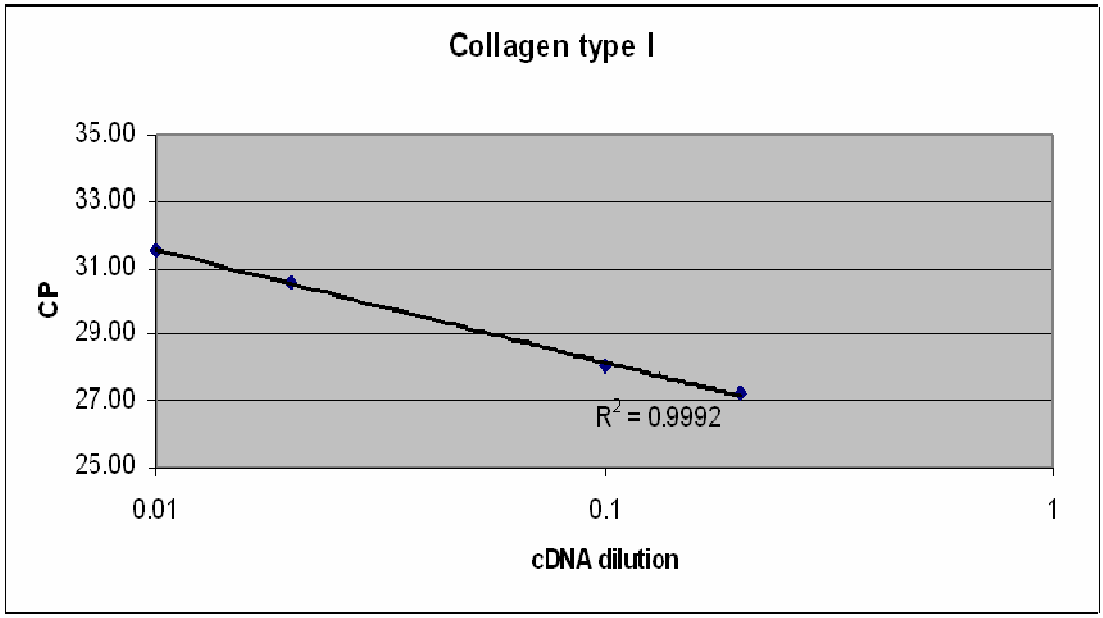
7.3.2.2 Cartilage Differentiation using 3D Pellet Culture

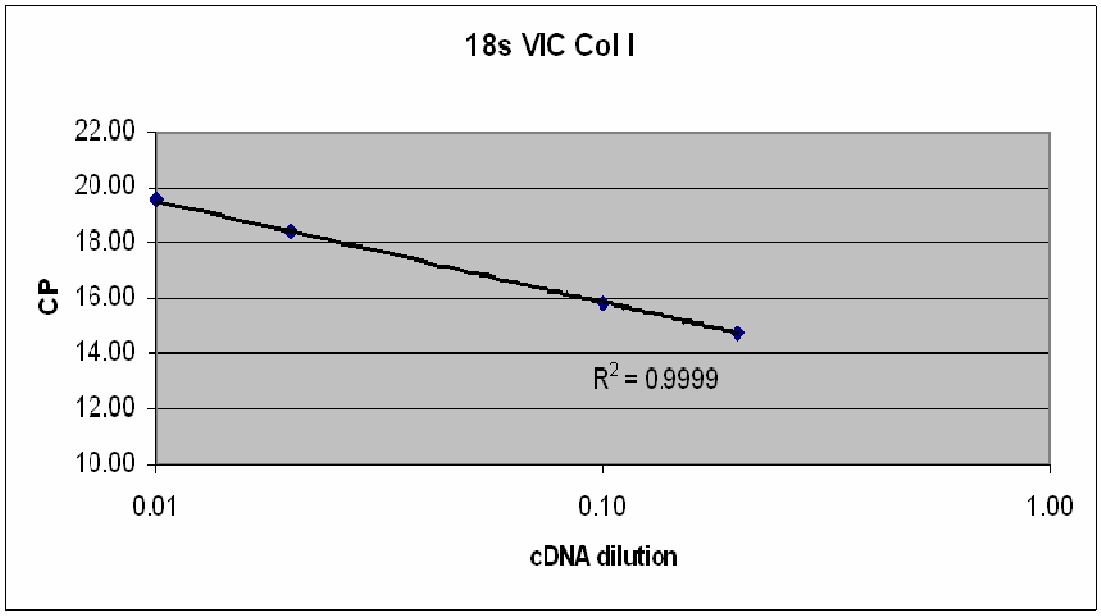
2 x 10^5 canine MSCs were placed into Falcon tubes and centrifuged to form pellet cultures. 24 hours following initiation spheroid pellets of approximately 0.5 mm were present in all differentiated and control Falcon tubes. Pellets remained present throughout the entirety of the experiment, however pellets cultured in standard MSC media tended to be less regular in shape, and showed some areas of cell breakaway and some cell growth present on the inner surface of the Falcon tube. Pellets grown in differentiated media appeared to shrink or contract and stayed spherical. At each time point pellets were taken for histological studies, and were used for RNA extraction. It was noted that over time the pellets in differentiation media became very hard, and were therefore difficult to break up to allow RNA extraction from cells. Those in standard MSC media were easily broken down and RNA extraction was therefore easier. This was reflected in total RNA extracted, with low levels of total RNA from pellets in differentiation media, particularly at later time points. Due to this difficulty in RNA extraction a repeat experiment was performed using 5 x 10^6 MSCs in the original start up pellet formation. These were then run through the full 21 day experiment and RNA extracted.

7.3.2.2.1 Quantitative RT-PCR Analysis of Cartilage Differentiation

7.3.2.2.1.1 Primer Optimisation

Primers for collagen Type I and II were optimized using RNA extracted from canine articular cartilage. R^2 values were calculated for both primer sets and 18s rRNA in house control gene which suggested high efficiency (Figure 7.10).


Collagen type I
35.00
33.00
31.00
29.00
27.00
25.00
CP
R2 = 0.9992
0.01
0.1
1
cDNA dilution


18s VIC Col I
22.00
20.00
18.00
16.00
14.00
12.00
10.00
CP
R2 = 0.9999
0.01
0.10
1.00
cDNA dilution

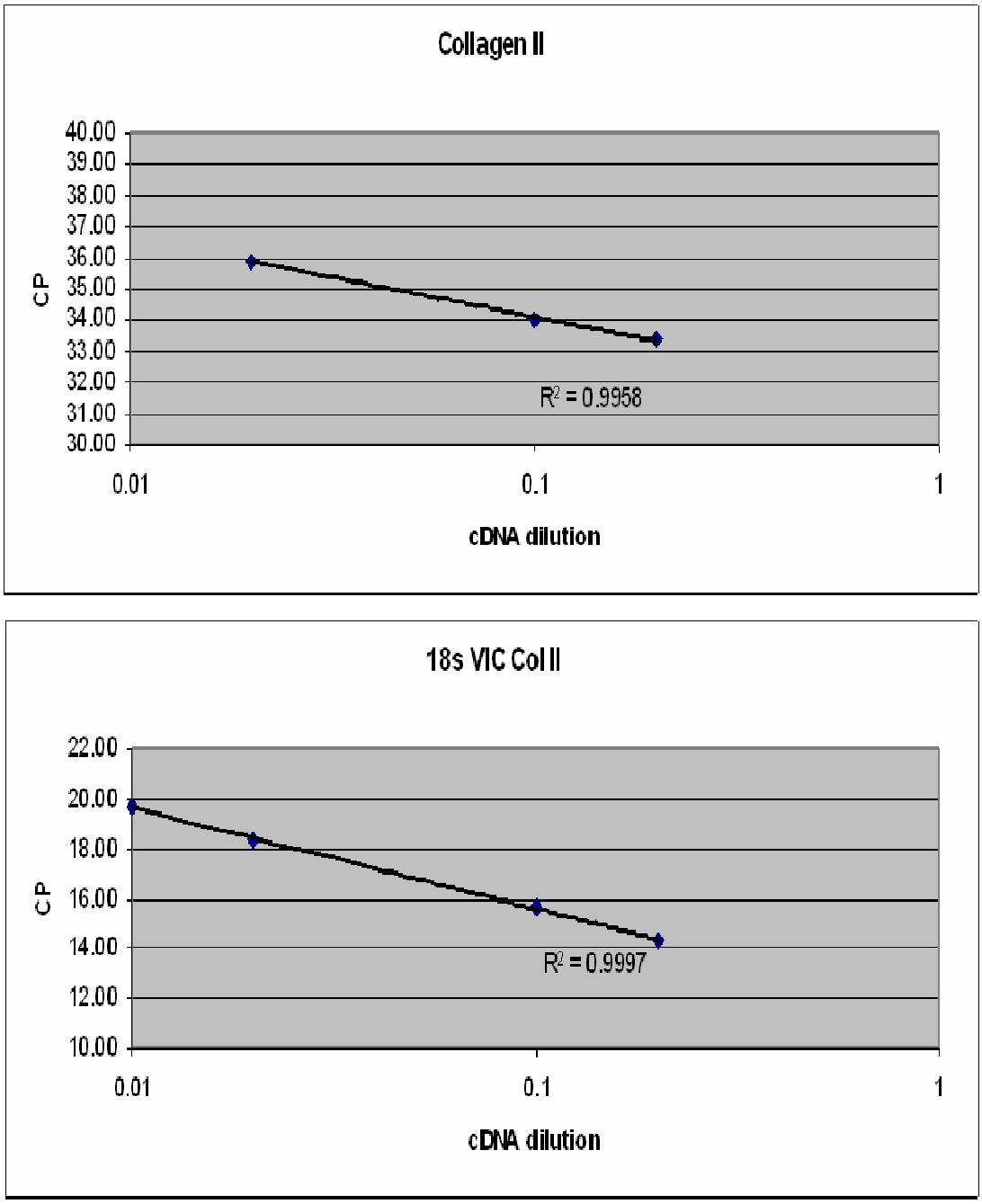


Figure 7.10. Primer optimization graph demonstrating R^2 values for collagen type I and II primers and their corresponding 18s rRNA primers using canine articular cartilage cDNA.

7.3.2.2.1.2 Relative Analysis of Collagen type I and II Expression

Following primer optimization relative expression of collagen type I and II was examined in two dogs using canine articular cartilage as a reference gene. For final data analysis sample duplicates and technical replicates were combined for each dog (Figures 7.11 and 7.12.

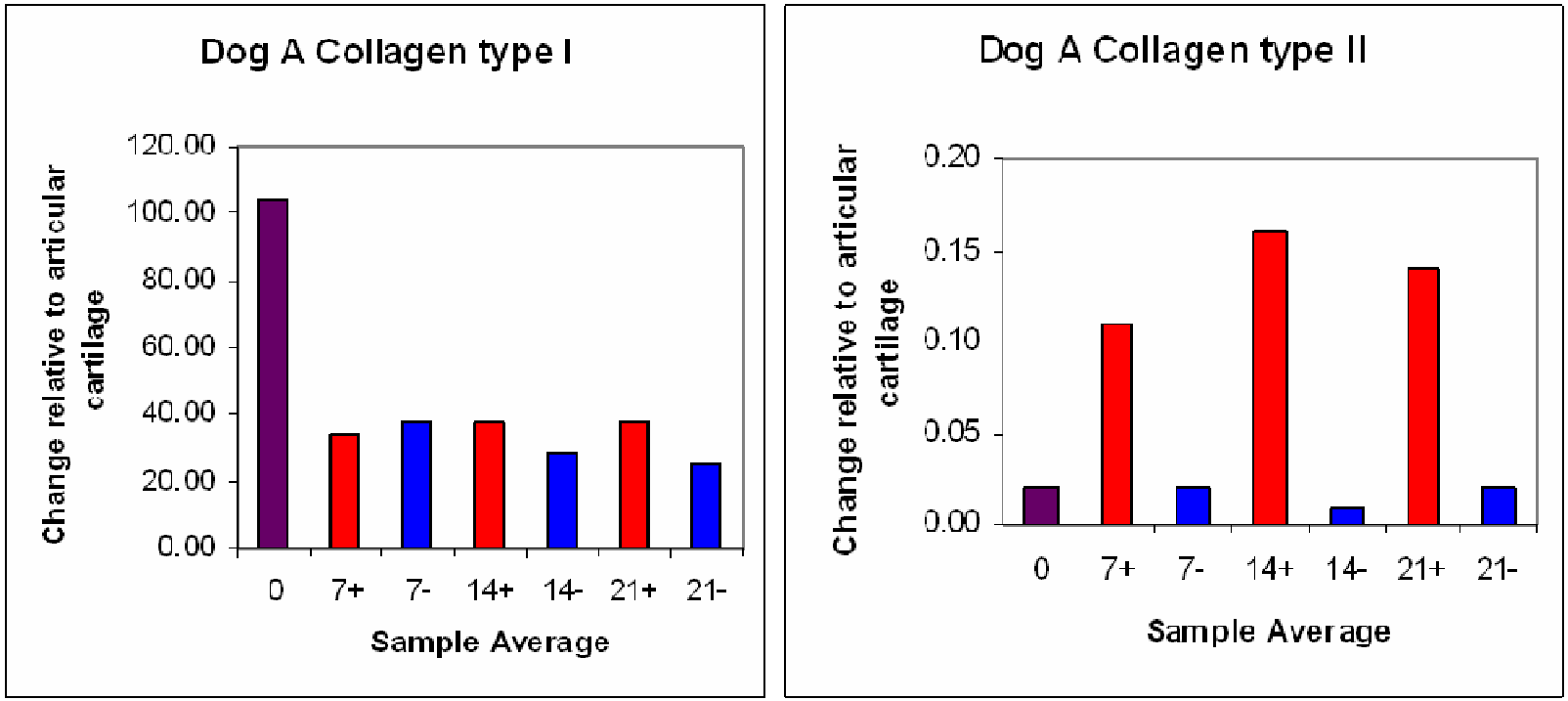


Figure 7.11. Quantitative RT-PCR analysis of marker expression showing fold change compared to articular cartilage of collagen expression in pellets from dog A over time (7, 14 and 21 days) in differentiated pellets (+) compared to controls (-).

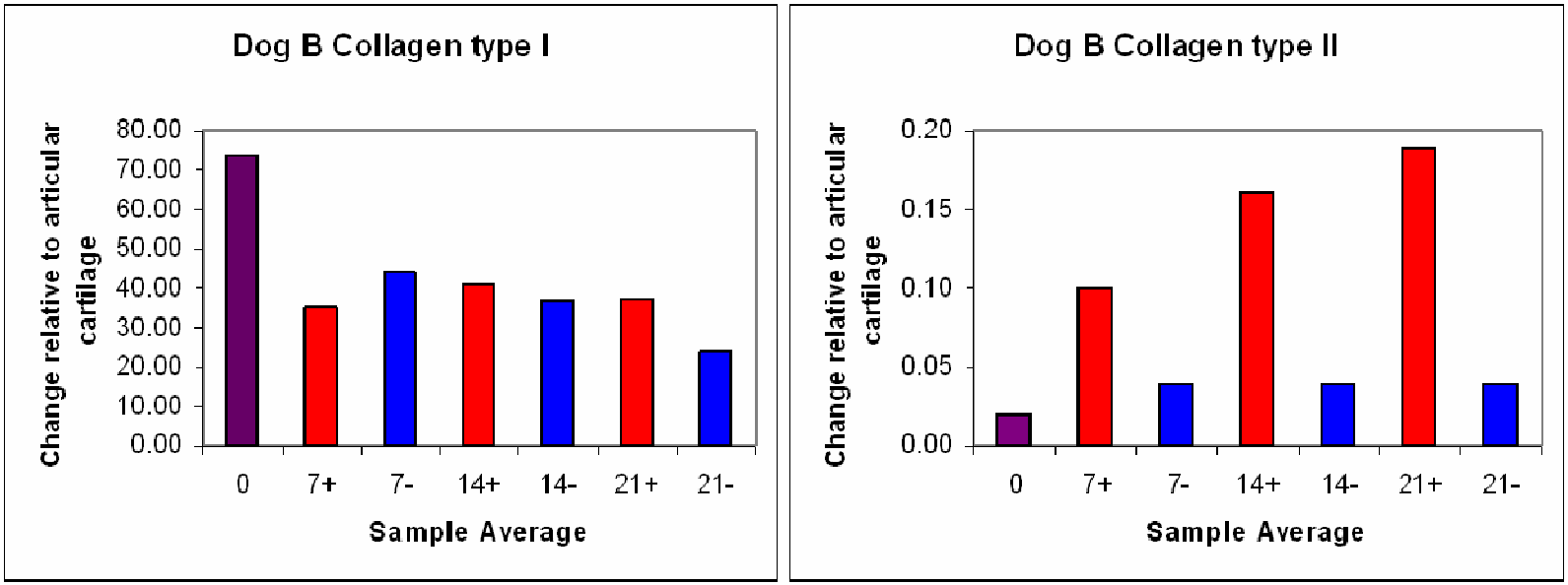


Figure 7.12. Quantitative RT-PCR analysis of marker expression showing fold change compared to articular cartilage of collagen expression in pellets from dog B over time (7, 14 and 21 days) in differentiated pellets (+) compared to controls (-).

In both dog A and B collagen type I expression was decreased approximately 50-60% in all samples compared to day 0 MSCs. Levels of collagen type I varied little across differentiated and undifferentiated samples across all time points. Dog A expression of collagen type II increased approximately 6 fold between day 0 and day 7 in differentiated pellets. In the undifferentiated pellets collagen type II remained at day 0 levels. At day 14 differentiated pellets increased expression to 8 fold that of day 0, which then returned back to 7 fold by day 21. In undifferentiated pellets of dog A collagen type II expression decreased to half that of day 0 levels at day 14 and returned to day 0 levels by day 21. Dog B increased expression of collagen type II by 5 fold at day 7, to 8 fold at day 14 and approximately 10 fold of day 0 levels in differentiated pellets. In undifferentiated pellets collagen type II increased 2 fold at all time points compared to day 0.

Pellet differentiation was repeated using an increase in MSC cell number at initiation. RNA was extracted and reverse transcribed and quantitative PCR performed as previously described (Figure 7.13). A small improvement was seen in total RNA extracted, allowing for 125 ng of total RNA to be used in reverse transcription to produce cDNA.

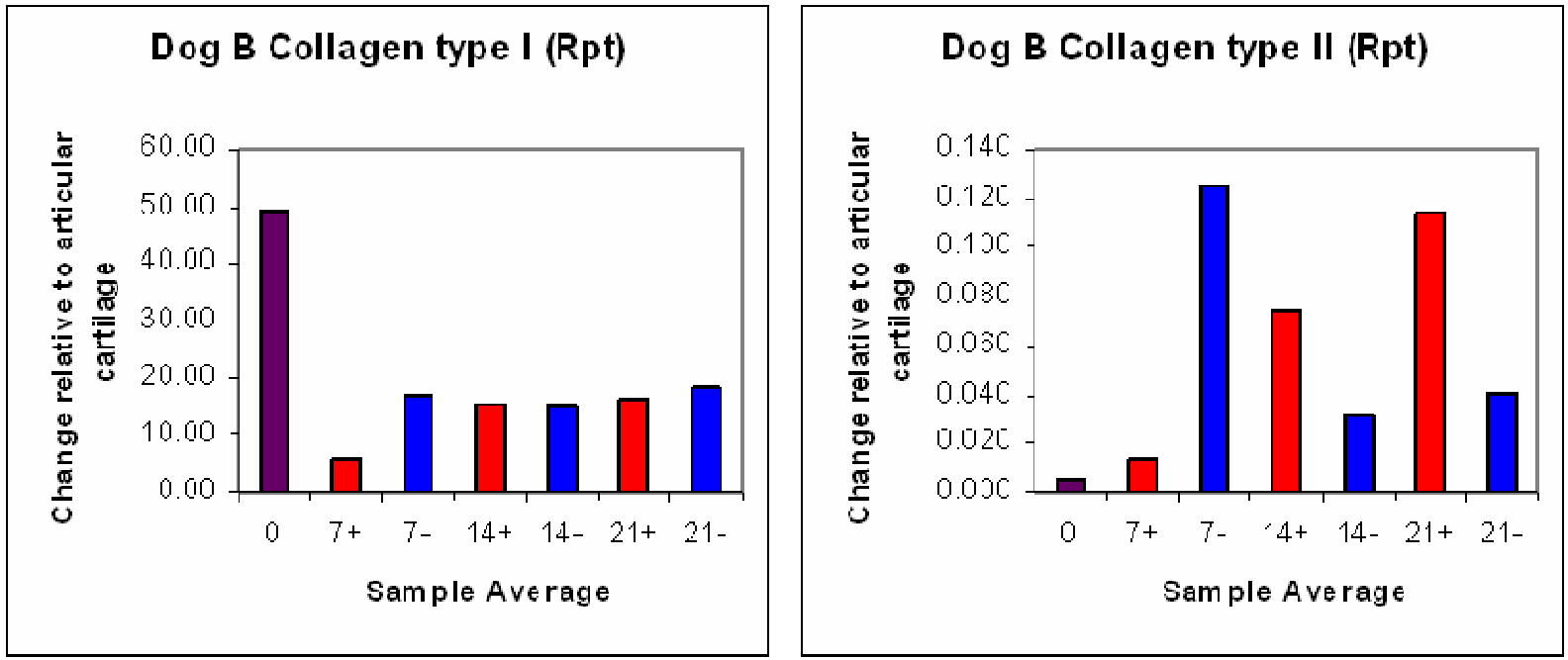


Figure 7.13. Quantitative RT-PCR demonstrating relative collagen expression in pellets with increased starting cell number from dog B at RNA level over time (7, 14 and 21 days) in differentiated pellets (+) compared to controls (-).

Collagen type I expression appeared similar to that seen previously. Expression decreased in all samples by approximately 60% when compared to day 0. However Collagen type II expression demonstrated less comparable findings. Day 7 differentiated pellets increased expression 2 fold compared to day 0, whereas day 7 undifferentiated pellets increased 20 fold. From day 14 expression patterns returned to those seen previously with day 14 differentiated pellets demonstrating a 12 fold increase in expression compared to day 0, which increased to a 19 fold increase at day 21. In the undifferentiated pellets there was an increase to 5 fold and 7 fold at day 14 and 21 respectively when compared to day 0.

7.3.2.2.2 Immunohistochemistry for Collagen Type II Expression

Cell pellets were examined for protein expression of collagen type II following differentiation. Initially positive and negative controls were performed using canine articular cartilage sections to optimize antibody dilutions (Figure 7.14).

Following optimization sections of differentiated and undifferentiated pellets were stained for collagen type II expression. Expression was seen extracellularly as would be expected and was seen in a diffuse pattern in differentiated pellets when compared to undifferentiated, where larger areas of non-staining were seen (Figure 7.15).

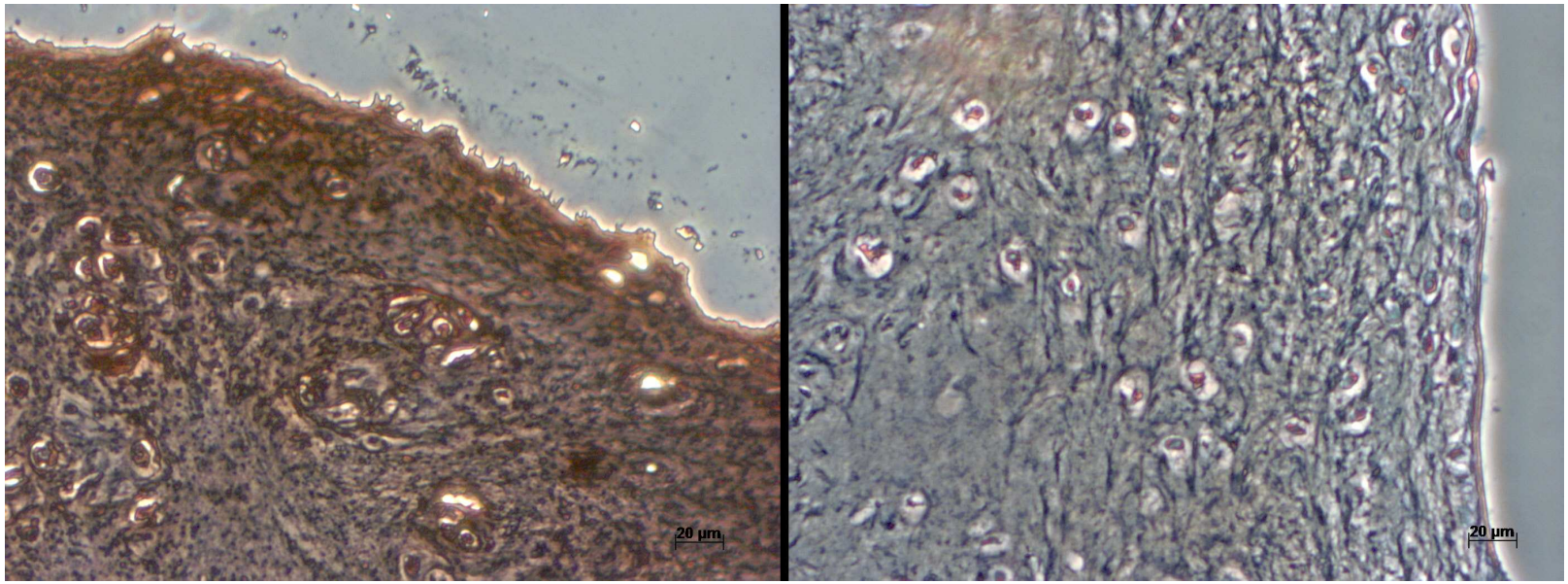


Figure 7.14. Collagen type II labelling of canine articular cartilage sections for antibody optimisation. Positive control (left) and negative control (right). Magnification x 400.

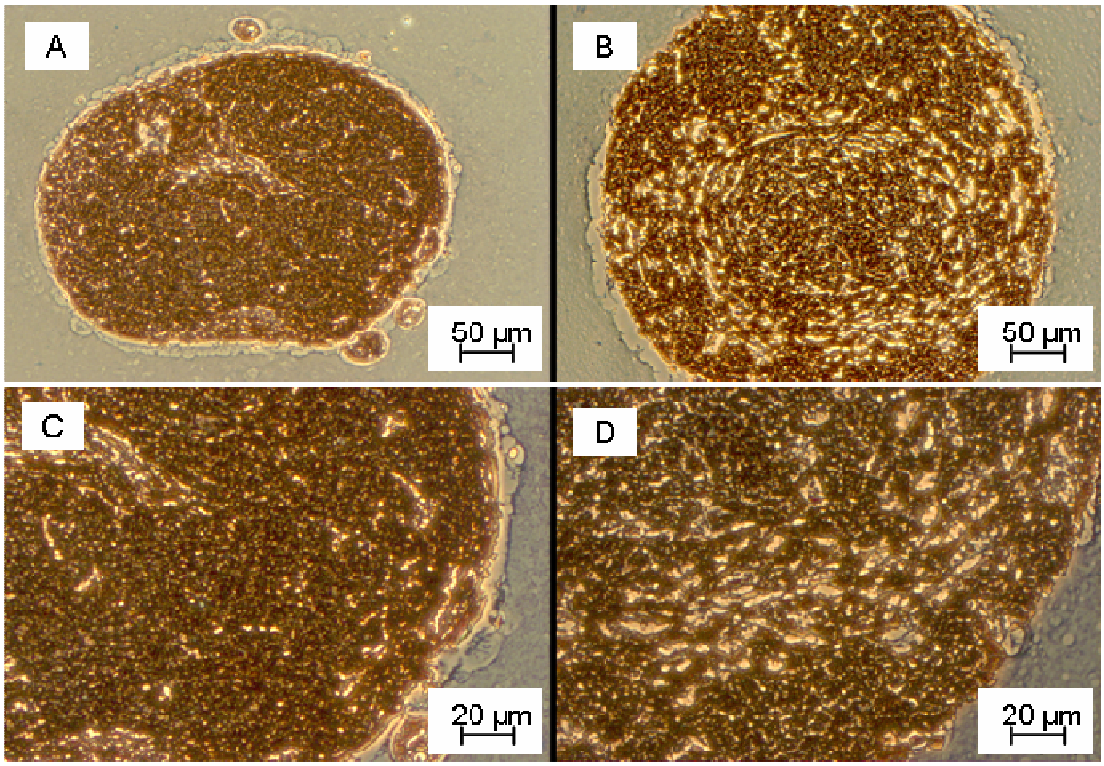


Figure 7.15. Collagen type II expression in differentiated (A and C) and undifferentiated pellets (B and D) with a more diffuse staining seen in differentiated pellets. Magnification x 200 A and B, x 400 C and D.

7.3.2.2.3 Immunohistochemistry for SOX9 Expression

Cell pellets were further examined for the expression of SOX9, a nuclear chondrocyte marker. Initially positive and negative controls were performed using canine articular tissue which demonstrated nuclear staining corresponding to SOX9 (Figure 7.16).

Following optimization differentiated and undifferentiated pellets were examined for expression of SOX9. A higher proportion of positive staining nuclei were seen in differentiated pellets when compared to undifferentiated and positive nuclei appeared regularly distributed throughout the pellet (Figure 7.17).

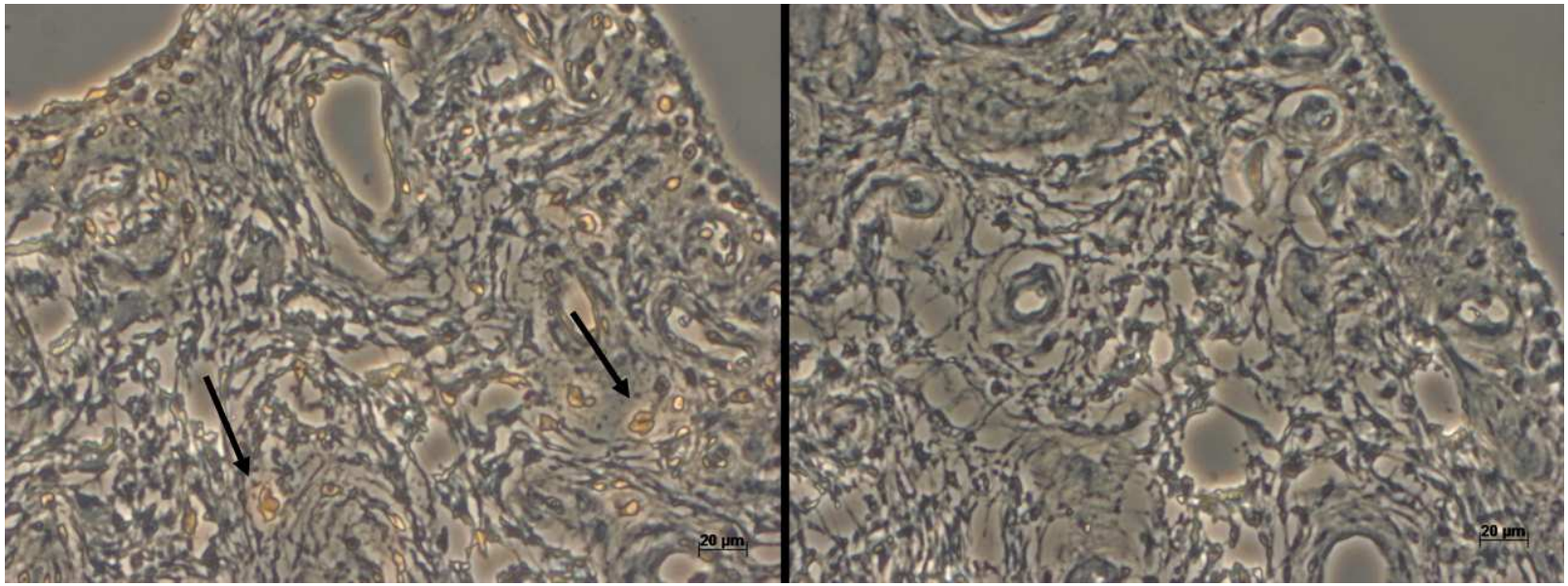


Figure 7.16. Nuclear SOX9 staining (black arrows) of canine articular cartilage sections for antibody optimisation. Positive control (left) and negative control (right). Magnification x 400.

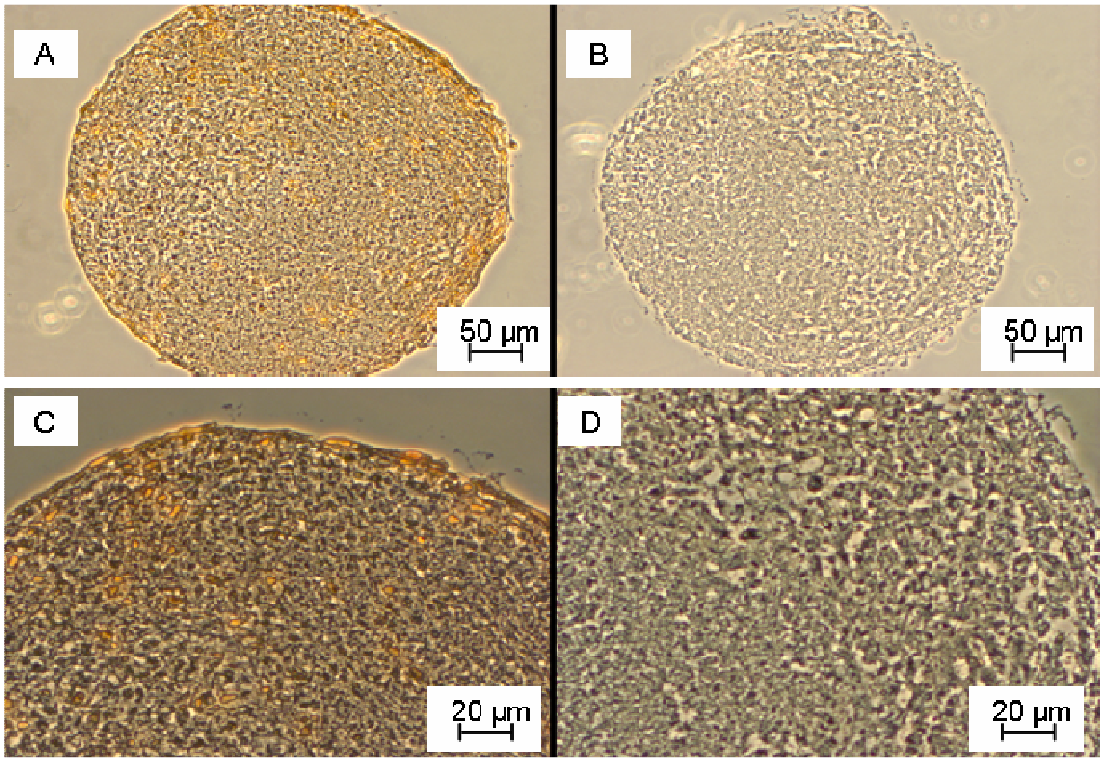


Figure 7.17. SOX9 expression in differentiated (A and C) and undifferentiated pellets (B and D) with nuclear staining seen in differentiated pellets. Magnification x 200 A and B, x 400 C and D.

7.3.2.2.4 Toluidine Blue Staining for Proteoglycan Matrices

Pellets were examined for toluidine blue which labels proteoglycan matrix. A steady increase in the uptake of toluidine blue was seen in differentiated pellets over time, which was not seen in corresponding undifferentiated pellets (Figure 7.18).

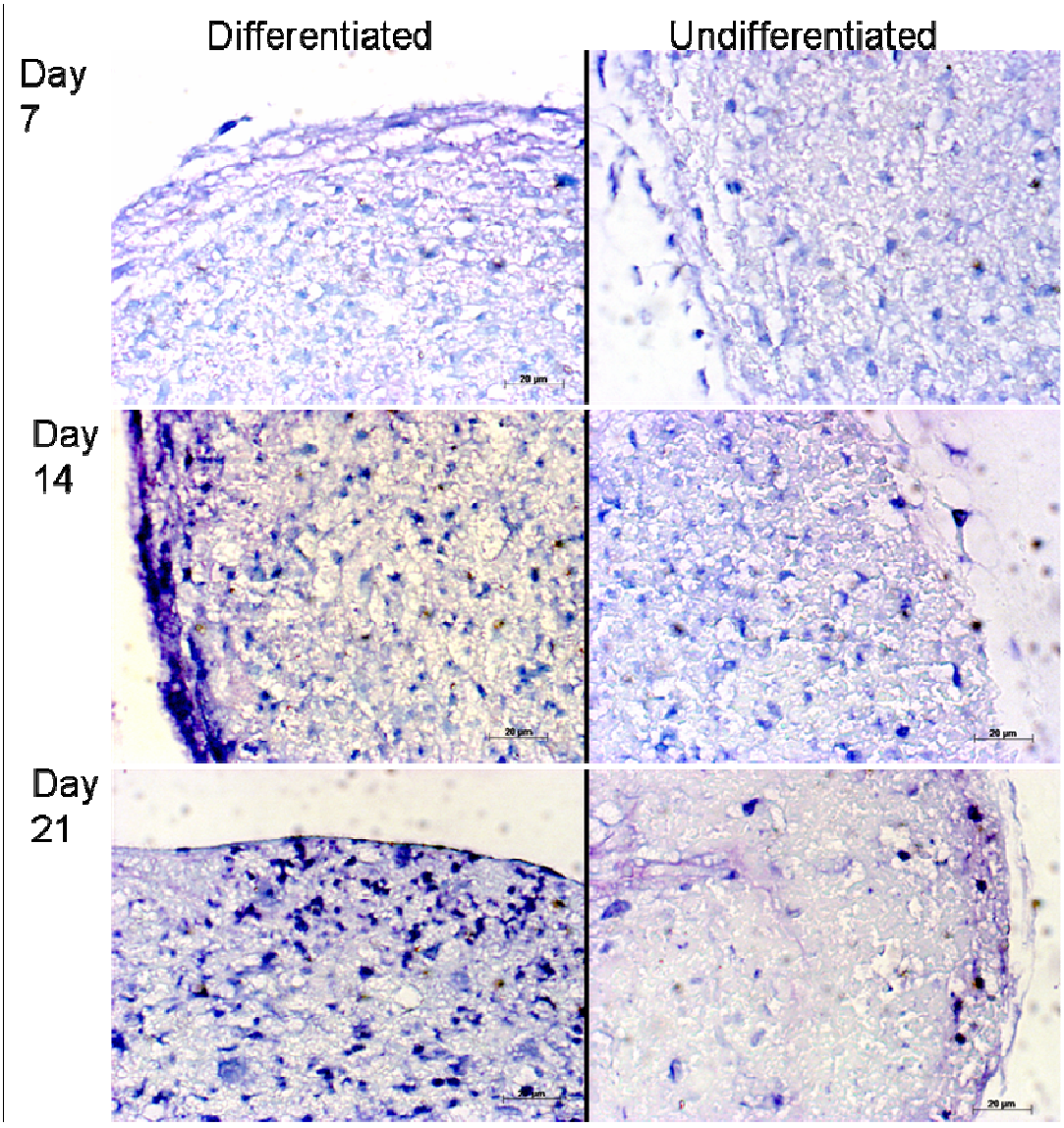


Figure 7.18. Toluidine blue staining of cartilage differentiation pellets. Pellets were examined for toluidine blue labeling at days 7, 14 and 21 for both differentiated and undifferentiated samples. Magnification x 400.

7.4 Discussion

Following standardized MSC isolation described in the literature MSCs should be capable of differentiation into adipocyte, osteoblast and chondrocyte lineages. Canine MSCs have previously been shown to be capable of differentiation into different mesodermal cell types (Kadiyala et al., 1997, Csaki et al., 2009) and we aimed to demonstrate the ability of our isolated canine MSC population to differentiate into cartilage.

7.4.1 Isolation and Culture of Canine Chondrocytes and Dermal Fibroblasts

Canine chondrocytes were easily cultured from isolated articular cartilage sections. These primary cells readily grew in culture and upon monolayer adherence lost their spherical shape and assumed a more fibroblast like morphology which may be suggestive of a switch from the articular type chondrocyte to a more fibrochondrocyte type which has been previously described (Benya et al., 1978, Stokes et al., 2002). Interestingly, evidence of extracellular matrix secretions were present on the surface of the flask during cell passage. This was a strong indicator of active chondrocyte presence, however given the morphological appearance of the chondrocytes in monolayer culture it would be likely that this secretion was predominantly collagen type I, plus extracellular matrix proteins (von der Mark et al., 1977, Kuettner et al., 1982).

Dermal fibroblasts were cultured as a negative control cell line for differentiation experiments. Following explant culture, cells of fibroblast morphology grew and replicated steadily. A mixed population of cells was present, with a predominant fibroblast phenotype and the indication of some adipocyte contamination. This is a common and likely occurrence with any primary sourced cell but must be taken into account when culturing cells for a particular cell type.

7.4.2 Are Canine Mesenchymal Stem Cells Capable of Cartilage Differentiation?

7.4.2.1 Differentiation of Canine Mesenchymal Stem Cells using Matrigel

It is known that chondrocytes in monolayer culture de-differentiate into fibrochondrocytes as previously mentioned. It is believed that chondrocytes cultured in suspension limits de-differentiation and therefore we aimed to differentiate canine MSCs within a semi-solid culture media using matrigel (Guo et al., 1989, Hauselmann et al., 1994). We cultured autologous chondrocytes and fibroblasts alongside to act as positive and negative controls respectively. However this experiment suffered two major setbacks. Firstly, to achieve semi-solid matrigel it must be used in a near undiluted form and this limited the amount of matrigel available (due to expense) and the experiment was designed to allow for this, leading to use of a 48-well plate and therefore low cell numbers. Wells were run in triplicate to try and allow for any inter-well variation and also to allow end point pooling if required. However, at first harvest no cells were seen

following pelleting and pooling using the cell recovery system. The aim had been to continue the experiment, and try harvesting cells again to obtain day 14 and 21 harvests, with the expectation that cell numbers would be higher given that replication would have occurred. However between day 7 and 14 we had a second setback where the plate became contaminated with fungus and the experiment was abandoned.

7.4.2.2 Differentiation of Canine Mesenchymal Stem Cells using 3D Pellet Culture

Using previously published pellet 3-dimensional culture and differentiation media (Yoo et al., 1998, Pittenger et al., 1999, Suzdal'tseva et al., 2007) we demonstrated that canine MSCs could be successfully differentiated into articular type cartilage. A steady increase in the articular related collagen type II over the three weeks of differentiation with a decrease in the more fibrous collagen type I expression was seen in differentiated canine pellets. Furthermore staining for SOX9 (a definitive chondrocyte nuclear marker) was increased and there was evidence of increased extracellular matrix secretions in the differentiated pellets compared to controls. This work compares favourably with previous work performed in both mouse and human MSCs where collagen type II was seen to increase, and collagen type I decrease following chondrogenic differentiation. (Lisignoli et al., 2005). Further research is required to elucidate the mechanisms behind cartilage differentiation and to optimize cell number. Published literature inputs 2×10^5 MSCs as a starting population (Yoo et al., 1998, Dennis et al., 2002) however this cell number is very small, leading to very small pellets and technical difficulties in RNA

extraction. When this cell number was raised a more irregular collagen type II expression increase was seen in differentiated cells leading to the conclusion that optimal cell number for successful differentiation is very small and this would be a hugely limiting factor if the aim was to use differentiated MSCs therapeutically.

7.4.3 Conclusion

This data demonstrates successful cartilage differentiation of primary sourced canine MSC, and furthermore that differentiation was selectively toward articular cartilage. This may have key significance in the future applications of MSCs for the study of canine orthopaedic disease *in vitro*. The use of MSCs in rebuilding damaged cartilage in osteoarthritis and articular defects will need to be optimized to allow for increase in cell number and to specifically analyse cartilage type when starting cell numbers are higher. The differentiation of MSCs using scaffolds is an exciting future area of research and may allow for more clinical applications of MSCs. Furthermore, investigation into the paracrine effects of MSCs (as discussed in Chapter 5 for cardiac disease) in joint disease is a developing area and may produce novel anti-inflammatory therapeutics.

CHAPTER 8

Final Discussion

This thesis set out to answer several questions regarding adult stem cells in the dog. Adult stem cells are a readily accessible potential source of multipotent cells, which bypass the technical and ethical issues surrounding canine ES cells, and are also considered safer in terms of therapeutic applications. Answers to the questions posed will have direct relevance to the field of stem cells and in particular veterinary disease study and therapeutics.

8.1 Can Adult Stem Cells be Isolated from the Dog?

We demonstrated successful isolation and culture of CSCs and MSCs from the heart and bone marrow respectively. The techniques used to obtain and grow these cells were based upon published literature for both the human and mouse, and we found that canine cells behaved similarly and particularly closely to human adult stem cells.

8.1.1 Cardiac Stem Cells

Canine CSCs have rarely been described (Linke et al., 2005, Bartosh et al., 2008) and we therefore undertook extensive characterisation of this isolated cell population. CSCs

could be harvested from the same explant tissue over several weeks based upon a technique described by Messina *et al* (Messina et al., 2004); typically 3-4 harvests could be taken before explants had to be discarded. We found that CSCs were able to grow in harsh serum free conditions, forming cardiospheres as described in the literature. Furthermore we found them capable of clonal expansion indicating their self-replication ability. CSCs were never obtained in significant numbers. Although multiple harvests could be taken from an explant and CSCs did replicate in culture as demonstrated using clonal expansion and sphere formation, they were a technically demanding cell population to work with. Furthermore they could not be frozen down; all attempts to do this resulted in dying cells upon re-plating. This meant that isolation of CSCs was totally reliant on fresh explant tissue, which was occasionally difficult to obtain and will therefore be a limitation of use of these cells in the future.

CSCs expressed key markers indicative of a cardiac progenitor line, such as c-Kit, GATA 4 and Flk-1 and were negative for cardiac lineage markers. As discussed in this thesis, there is a belief that multiple stem cell populations reside within the adult heart defined by developmental markers such as islet 1, Nkx2.5 and Flk-1. We were unable to isolate an islet 1 positive cell line consistently, and this is an area of future research. Isolation of islet 1 populations has been performed in rodent models using cre-lox technology which enabled isolation of living cells which could then be used for downstream applications (Laugwitz et al., 2005). This is obviously not possible yet in the canine, and therefore novel techniques will need to be devised. Initially a study to examine presence and location of islet 1 in the myocardium should be performed, using

cardiac sections and techniques such as immunohistochemistry or in situ hybridisation. This would be a laborious and time consuming project given the likelihood of low islet 1 positive cell numbers, but would be rewarding should the data indicate a region of the heart where islet 1 cells predominate. In this project CSCs were found to be isolated in higher number from atrial explants than ventricular, and therefore early in the project a decision was made to work solely with atrial explants for CSC isolation. This subjective observation may have in fact been due to stem cell niche locations as discussed previously (Urbanek et al., 2006), but it must be acknowledged that this may have skewed the CSC population we isolated and experimented with.

8.1.2 Mesenchymal Stem Cells

Canine MSCs have been described in more detail than CSCs in the literature, however we still undertook extensive characterisation of our cells and again found that they compared favourably with human MSCs (Dominici et al., 2006). Canine MSCs were CD44 and STRO-1 positive, negative for CD45 and predominantly negative for CD34. They were readily isolated from the bone marrow using Ficoll separation, and were obtained in high number. Canine MSCs adhered naturally to tissue culture plastic flasks allowing for contaminating haematopoitic cells and debris to be removed using media washes. The cells replicated rapidly, and could be harvested and frozen down and used for later experiments. This meant that canine MSCs were easily expanded and multiple experiments could be performed using cells from the same dog if required. Canine MSCs did however senesce between passage 4-6, with significant slowing of cellular

replication and alteration of morphology with increased size. No late passage cells were used for experiments, and this senescence compared favourably with primary human MSCs (Banfi et al., 2000, Sekiya et al., 2002). However, this behaviour does bring into question the stem-like properties of canine and human MSCs, and whether 'stemness' is lost over time in culture. The long term culture characteristics and marker expression of canine MSCs were not analysed in this thesis, and would be something worthy of investigation. Based upon that, further research to prolong the replicative capacity and maintain primary MSC expression profiles would be useful.

Using Ficoll separation and tissue culture plastic adherence allows for a more heterogeneous MSC population to be isolated initially. We acknowledge this, and allowed for it to occur to therefore not select against a population of cells which may in fact be more stem-like than another. It is likely that several different MSC populations reside within the bone marrow, each with their own characteristics and differentiation potential and future work could investigate cell sorting for isolation of individual MSC populations and analysis of their characteristics. Magnetic Activated Cell Sorting (MACS®) and Fluorescent Activated Cell Sorting (FACS) would be ideal techniques to do this, but would rely upon cell surface markers for sorting. Care must be taken when performing these experiments due to marker expression changes during cell isolation and processing; for instance certain cell surface receptor epitopes may be blocked by proteins when in serum which could skew results and affect sorting efficiency. MACS and FACS are also reliant upon antibody selection, and as discussed in this thesis dog specific antibodies are rare and therefore specificity must be validated.

Alternative MSCs populations were demonstrated in this thesis by the discovery of a CD34 positive MSC population from the canine bone marrow. Up until that point all MSCs had been CD34 negative, which corresponded to standard MSC characterisation profiles. CD34 is a cell surface adhesion marker, but can also be used to indicate haematopoietic lineages (Nielsen and McNagny, 2008). Given these CD34 positive cells were CD45 negative it may indicate an MSC population with both mesodermal and haematopoietic differentiation potential. These cells were discovered late on in the project, and therefore time did not allow for extensive work upon them. However, this is certainly an area for future research, either with the specific cells isolated (which have been frozen down as stocks) or by selecting for CD34 from the original bone marrow harvest.

8.1.3 VEGF Splice Variation in Stem Cell Populations

VEGF gene expression was seen with a multiple banding pattern representing splice variation. This difference in banding is representative of the production of the different VEGF isoforms, and as discussed these isoforms are known to have different functions (Poltorak et al., 1997). Interestingly both MSCs and CSCs had the same banding pattern, which may indicate that this pattern is stem cell specific. Furthermore, this pattern was different to that seen with differentiated tissues such as the whole heart tissue used as a control sample. The relevance of the banding pattern and the role of the individual

isoforms in stem cells and somatic tissues in the dog is an area which will need further investigation.

8.2 Can Canine Adult Stem Cells be Differentiated Toward Somatic Cells?

8.2.1 Cardiac Directed Differentiation

This project focussed heavily upon cardiac aspects of adult stem cells due to the need for more veterinary research in this area. We therefore took both CSCs and MSCs and differentiated them toward cardiac lineages using techniques described in the literature (Oh et al., 2003, Kruglyakov et al., 2006, Smits et al., 2009b).

CSCs appeared to differentiate toward a cardiomyocyte lineage, with upregulation of the cardiac specific transcription factor Nkx2.5 and the cardiac functional gene CTT. We defined this differentiation as partial. No other cardiomyocyte specific markers were upregulated such as CTI, the ryanodine receptor and the β_1-adrenergic receptor. Furthermore no evidence of spontaneous rhythmical beating was seen. This was frustrating, and further work will need to be performed to improve on the current differentiation techniques. Co-culture with RNCM may be a valid option, and one we investigated as a possibility. However, due to the technical difficulties in obtaining CSCs in terms of timing, it was difficult to obtain RNCM at the precise time CSCs were ready for harvest. Also this technique would require direct co-culture which would therefore make analysis more challenging, although not impossible; for instance,

RT-PCR primers could be designed specifically for the canine, thereby not picking up rat transcript, and translational analysis such IFA could use antibodies specific for canine protein. Should techniques to isolate specific cardiac progenitor populations be developed as discussed above, it would be interesting to repeat cardiac differentiation with the individual progenitor cell types to see if more efficient differentiation occurs with one particular line more than another.

Canine MSCs showed no evidence of cardiac directed differentiation, even when using techniques claiming successful differentiation in the literature. MSCs are from the mesodermal lineage, as are cardiomyocytes, and therefore it was hypothesised that there is an inherent nature of MSCs to move toward cardiac lineages. Minor morphological changes were seen in differentiated cells, with suggestion of lining up. We propose that this may indicate some level of cellular organisation, however no evidence in terms of marker expression supports this and therefore this result is subjective. Nkx2.5 expression was seen to increase, similar to the CSCs but this marker in isolation cannot be used to determine cardiac differentiation. The combination of morphological changes and increase in Nkx2.5 may indicate a very early stage of differentiation, and may warrant the investigation of improving the current differentiation techniques.

Flk-1 expression was seen to decrease in CSCs and increase in MSCs following cardiac differentiation. Flk-1 is both a cardiac developmental marker, and also an endothelial marker due to its nature as a VEGF receptor. This difference in Flk-1 expression may represent a movement away from endothelial lineages when reduced, and vice versa. It

would be interesting to follow this result up further, and examine whether MSCs are in fact moving toward a cardiac endothelial lineage rather than cardiomyocyte as we have proposed for CSCs.

8.2.2 Cartilage Directed Differentiation

Canine MSCs were differentiated toward cartilage based upon the literature stating that MSCs should be capable of multiple connective tissue type differentiation (Dominici et al., 2006). Canine MSCs were found to differentiate to articular type cartilage, using standard techniques, thereby validating that we were isolating the correct cellular population from the bone marrow. This positive result is exciting for future orthopaedic applications of MSCs and could be used for the development of *in vitro* disease modelling. However, we found that very small cell numbers were optimum for differentiation to succeed and therefore investigations into maximising cell number needs to be performed to allow for further downstream applications and even clinical applications. An interesting area of research is the development of scaffolds for cartilage differentiation, particularly those which can be used therapeutically and the investigation of our canine MSC populations and their differentiation capability on these scaffolds would be worthwhile.

8.3 Do Rodent Models Compare Favourably with the Dog?

Due to the poor cardiac directed differentiation of canine MSCs and given that the literature claiming successful differentiation was based upon rodent models (Makino et al., 1999, Fukuda, 2001, Kruglyakov et al., 2006), it was deemed worthwhile to purchase murine MSCs to run alongside the dog cells as a positive control. We designed mouse specific primers, and analysed the cells extensively for our full panel of MSC and cardiac markers. At initial characterisation mouse and canine MSCs appeared morphologically similar. However striking differences appeared between culture behaviour and marker expression, with murine MSCs capable of long term passage and expressing several cardiac lineage markers such as CTT, CTI and the ryanodine receptor. This immediately brought into question the similarities between canine and murine MSCs. Furthermore, differences in marker expression seen during cardiac differentiation appeared to be explained by time in culture alone. Based upon that, we analysed early and late passage mouse MSCs for our marker panel and found an increase in the expression of the cardiac markers CTT, CTI, islet 1 and GATA 4 and a downregulation of SMA. These results taken in isolation could infer cardiac differentiation, however no significant morphological changes or rhythmical beating were observed, therefore cardiac differentiation is unlikely to have occurred. It may be that the expression of these markers indicates a pre-determined cardiac lineage potential which is greater than that of the canine MSCs. However techniques to improve differentiation need to optimised and the results seen here reiterate the importance of extensive baseline analysis of cells prior to differentiation.

8.4 Does Stem Cell Co-Culture Influence Cellular Expression?

We devised a novel indirect co-culture experiment between autologous CSCs and MSCs to investigate the role of released factors upon each cell population. The paracrine effects of stem cells, and in particular MSCs is an exciting new area of research, and investigates whether adult stem cells may be useful not only for cellular replacement and *in vitro* studies but also for their released factors modulating tissue environments. The co-culture experiment was technically challenging due to the need for both CSCs and MSCs to be ready for experimentation at the same time when harvested from the same dog. We ensured no cellular crossover could occur by using small diameter transwell inserts, and analysed marker expression from both cell populations. Interestingly the CSCs appeared to show a morphological change toward an endothelial cobblestone appearance, which was combined with an apparent increase in Flk-1 and vWF expression. This may imply that MSCs release factors which direct CSCs toward the endothelial lineage and not toward the cardiomyocyte. In the reverse experiment there was no significant alteration in MSC expression toward any of the cardiac lineages. The affect of the MSCs upon the CSCs will need to be investigated further, in particular protein analysis of soluble released factors in the media and their function.

During the co-culture experiment we discovered the canine CD34 positive MSC line. CD34 was seen to switch off in MSCs co-cultured with CSCs. The cells in the co-cultured wells peeled off into 3D pellets at early stages, which was not seen in

control wells even though cell numbers where the same at input. CD34 is a cell adhesion molecule, so the downregulation of this marker may have triggered the cellular release from the base of the well thus forming the free floating pellet. Furthermore CD34 in mice has been found important for angiogenesis although the importance of that here is unknown. The results do suggest that CD34 was downregulated in response to the co-culture environment with CSCs and this interesting finding is an area of future research, again looking at the soluble released factors from CSCs and relevance of both the released factors and CD34 expression in canine MSCs.

8.5 Conclusion

This thesis demonstrates the isolation and characterisation of two adult stem cell sources from the dog. These cells conformed to stem cell criteria and were capable of differentiation along different routes and offer a potential new source of cells to study veterinary diseases *in vitro*. The results obtained have answered the initial questions posed, and several others which became evident during the research. The results have also led to the development of new questions which will require future investigations and the beginning of new projects. The field of stem cell research is constantly developing and evolving, and the hope is that the data produced here will contribute to this field in a positive way.

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Appendix A;

Published Papers

1. Hodgkiss-Geere, H.M., Argyle, D.J., Corcoran, B.M., Whitelaw, B., Milne, E., Bennett, D., Argyle, S.A., 2012. Characterisation and cardiac directed differentiation of canine adult cardiac stem cells. The Veterinary Journal 191, 176-182. PLUS Guest editorial comment on this paper

2. Hodgkiss-Geere, H.M., Argyle, D.J., Corcoran, B.M., Whitelaw, B., Milne, E., Bennett, D., Argyle, S.A., 2011. Cardiac Specific Gene Expression Changes in Long Term Culture of Murine Mesenchymal Stem Cells. International Journal of Stem Cells 4, 143-148.

Appendix B;

Published Abstracts

1. **Characterisation and Cardiac Directed Differentiation of Canine Adult Stem Cells. British Small Animal Veterinary Association Congress, Birmingham, UK. April 2010.**

2. **Canine Adult Stem Cells as a Model for Human Cardiac Stem Cell Research. World Stem Cell Summit, Detroit, USA. October 2010.**

Characterisation and Cardiac Directed Differentiation of Canine Adult Stem Cells

Introduction

Cardiac disease is one of the most significant causes of morbidity and mortality in dogs, cats and humans. Until relatively recently the heart was considered a terminally differentiated organ, however it is now thought that the heart has a resident population of adult stem cells (ASCs) which may be capable of some degree of self-repair.

ASCs are capable of multipotent differentiation, and are lower in the stem hierarchy. They have been isolated from several organ systems, and described in multiple species. The dog may act as a large animal disease model, and is also a clinical subject in its own right. Development of organ specific stem cell culture may act as an animal sparing procedure for pharmaceutical testing. Therefore we have aimed to isolate and characterise adult stem cells from the dog.

Materials and Methods

Atrial cardiac explants and bone marrow aspirates were taken from dogs post mortem and cultured using previously published techniques. Putative stem cell harvests were collected from these tissues, and cardiac lineage directed differentiation was performed. Gene expression profiles of stem cell markers, mid-differentiation markers and cardiac differentiated markers were investigated at both the RNA and protein level to determine outcome of the differentiation procedure.

Results

Phase bright cells isolated from cardiac explants demonstrated similar behaviour to those described in the literature for cardiac stem cell populations. These cells were able to survive successive passages in serum free media and often formed large spherical cell clusters, termed ‘cardiospheres’. These cells were capable of clonal expansion under controlled culture conditions, demonstrating their ability for self-renewal. Cells cultured from the bone marrow behaved typically as mesenchymal stem cells, and following cardiac differentiation appeared morphologically altered, with cells forming alignments when compared to controls.

Conclusions

Our results suggest that it is possible to isolate adult stem cells in the dog from both the mesenchyme and the heart itself. These cells are capable of clonal expansion, and directed differentiation. The combination of clonal expansion and gene expression profiles supports and justifies further characterisation of these cell populations. Characterisation of these cells is crucial in furthering our understanding of their biology and potential; adult stem cells have provided great hope in the development of regenerative medicine and tissue engineering.

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Canine adult stem cells as a model for human cardiac stem cell research

Problem: Cardiac disease is a significant cause of morbidity and mortality in humans and dogs. Although rodent modelling systems allow for cost effective and rapid analysis the results obtained rarely directly translate into human disease systems. Larger animal models are required to study cardiac stem cell biology.

Background: The dog is a species which develops naturally occurring cardiac disease; including clinical syndromes seen in humans. Moreover the pet dog is a close human companion, and therefore shares common environmental factors of disease.

Hypothesis: Adult stem cells have been isolated from the heart in both mice and humans, which have been found to be multipotent and clonogenic. We propose that the dog also has a resident cardiac stem cell population, which can be isolated, characterized in terms of marker expression profiles and behaviour following cardiac directed differentiation allowing direct comparisons with human cardiac stem cell populations.

Research: Using published techniques, atrial cardiac explants were taken from dogs *post-mortem* and cultured to isolate putative adult stem cells. These cells were characterized using an extended panel of markers, and compared to those expressed in human populations. The canine cells were subsequently placed into cardiac lineage differentiation protocols, and the expression profiles analyzed.

Observations: Following isolation from cardiac explants, large round phase bright cells were harvested and placed into stem cell culture. These cells were able to survive successive passages in serum free media and formed large spherical cell clusters, termed ‘cardiospheres’. These cells were capable of clonal expansion under controlled culture conditions, demonstrating their ability for self-renewal. Characterization of marker profiles demonstrated c-kit, GATA 4 and flk-1 positive cells which were Nkx2.5 and cardiac lineage marker negative. Following cardiac directed differentiation marker profiles altered with upregulation of cardiac troponin T and Nkx2.5 and a down-regulation of c-kit and endothelial lineage markers.

Conclusions: From our observations we have found a cell population that can be isolated from the canine heart and which behaves similarly to previously described human cardiac stem cells. Furthermore, we have demonstrated a change in marker expression consistent with cardiac directed differentiation. Further work will target isolating and characterising further adult stem cell populations from the dog, and exploring their differentiation potential. We conclude that canine adult stem cells are directly comparable to human stem cells, and are therefore an appropriate large animal modeling system for cardiac stem cell research.

Appendix C;

List of Conference Presentations and Posters

6th – 8th April 2009

Association of Veterinary Teachers and Research Workers Annual Conference. Scarborough, UK. Presented 'Isolation and Characterisation of Canine Adult Cardiac Stem Cells'

26th & 27th August 2009

North East Stem Cell Institute – Adult Stem Cells in Regenerative Medicine and Disease. Newcastle University, UK. Presented talk and poster 'Isolation and Characterisation of Canine Adult Cardiac Stem Cells

13th & 14th November 2009

Veterinary Cardiovascular Society Annual General Meeting. Loughborough, UK. Presented 'Isolation and cardiac differentiation of adult stem cells from the dog'

7th April 2010

British Veterinary Orthopaedics Association. Birmingham, UK. Presented 'Stem Cells for Canine Joint Disease'

8-11th April 2010

British Small Animal Veterinary Association Congress. Birmingham, UK. Presented 'Characterisation and Cardiac Directed Differentiation of Canine Adult Stem Cells'

4th – 6th October 2010

World Stem Cell Summit, Detroit, USA. Presented poster 'Canine Adult Stem Cells as a Model for human Cardiac Stem Cell Research'

1st July 2011

UK Mesenchymal Stem Cell Meeting, Aston University, UK. Presented poster ‘Characterisation, differentiation potential and comparisons of two mesenchymal stem cell populations’